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Controlling Maillard Pathways To Generate Flavors

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Donald S. Mottram, Editor
University of Reading

Andrew J. Taylor, Editor
University of Nottingham

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Foreword

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Preface

The role of the Maillard reaction in forming flavors from amino acid and sugar precursors has been studied for many years. To establish the basic chemistry of the reaction, researchers have used model systems, often solutions of a single amino acid with a single sugar. Despite the apparent simplicity of the system, heating such a solution can generate tens if not hundreds of compounds and this requires careful and time-consuming analysis to identify and quantify each component. This task is especially laborious if the Maillard reaction is followed over its time course (or under different processing conditions, e.g., temperature or pH) when many analyses are required.

Data from the model systems has allowed researchers to study the pathways that lead to flavor formation, and various schemes have been proposed to identify the main "routes" that lead to flavor compounds. The scheme proposed by Hodge (see Chapter 14 for references) was published many years ago but still gives a good overall view of the pathways. Such schemes have led to one of the main control principles, namely an understanding of the role of amino acids in forming some characteristic aromas, e.g., bread flavor from proline, as well as an appreciation of the role of C5 and C6 sugars in controlling the rate of reaction.

The main development since the publication of model system reaction schemes has been to understand the pathways in greater detail. Isotopic labelling of sugars and amino acids with ^{13}C and ^{15}N has proved highly effective in identifying the origin of Maillard end-products and has shown that, in some cases, an end-product can be formed by more than one pathway. Similarly the possibility that reactants other than sugar and amino acids can take part in the Maillard reaction has been demonstrated and the role of carbonyl compounds formed from lipid oxidation in the development of meat flavor has been clearly established. More recently, the formation of taste compounds through the Maillard reaction has been investigated and new potent compounds have been discovered that can contribute to the overall flavor formed during the Maillard reaction. These findings also offer the potential for control and manipulation of the Maillard reaction to form specific types of flavor.

Although the nature of the end-products of the Maillard reaction in both food and model systems are well documented, applying these principles to control flavor formation in real foods has proved difficult. Some of the challenges are recognized and well-documented. For instance, different parts of food materials receive different thermal treatments during processing (e.g. baked bread), which causes the Maillard reaction to occur to different extents on the surface and interior of foods. Foods can also contain many different types of amino acids, as well as several different sugar sources, and the reactivity of mixtures of amino

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acids creates a further level of complexity. The ways in which the physical and chemical parameters of the food matrix can also influence the mobility and availability of reactants (and hence change the flavor characteristics in foods) is also recognized but not so well researched as other aspects.

This book is based on a symposium held at the 238th ACS National Meeting in Washington DC, August 2009, and describes recent research and developments related to the control of the Maillard reaction to give optimum flavor quality. These include kinetic modeling of the reaction, the effect of physical parameters (temperature, time, moisture content, pH), and the effect of chemical parameters (amino acid and sugar composition, the presence of other components). The topics covered relate to real food systems and reaction product flavorings, as well as model systems. Contributors from academia and industry have come together to provide an up to date overview of progress in this important area of flavor research.

The other purpose of the symposium was to honor the work of Professor Don Mottram from the University of Reading, UK on the occasion of his retirement. He has been active in Maillard chemistry for many years and has been responsible for some seminal work, such as the role of lipids in meat flavor formation and the mechanism for acrylamide formation in heated foods to mention just two. He has also been a great mentor to flavor researchers and has been responsible for training and encouraging many people who have gone on work in both fundamental and applied flavor research. We thank him for his contribution to Maillard flavor research and his continuing enthusiasm for the subject.

Andrew J. Taylor

Division of Food Sciences
University of Nottingham
Sutton Bonington
Loughborough LE12 5RD
United Kingdom

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Chapter 1

Predictive Modeling of Flavor Compound Formation in the Maillard Reaction: A SWOT Analysis

M. A. J. S. van Boekel*

Department of Agrotechnology and Food Sciences, Wageningen University,
P.O. Box 8129, 6700 EV Wageningen, The Netherlands
***Tiny.vanBoekel@wur.nl**

The importance of Maillard flavor compounds for food quality is undisputed, but we are far from being able to control such formation quantitatively in food processing. Kinetic models attempt to predict rates of formation as a function of temperature, pH, water activity/content, and chemical reactivity. There is, however, a gap between mechanistic models for well-defined model systems, and their application to food, where we are left mainly with empirical models. This paper addresses the *strengths* and *weaknesses* of the current models in their ability to predict Maillard flavor compounds quantitatively, the *opportunities* to progress with these models to make the step to real foods, and the *threats* that models are simplifications by their very nature. Topics addressed are the usefulness of uniresponse *versus* multiresponse models, mechanistic *versus* empirical models, model systems *versus* real foods. A combination of kinetics and thermodynamics is proposed as direction for future research.

Introduction

This paper is about the ins and outs of kinetic modeling of the Maillard reaction, with an emphasis on the flavor compounds formed. It is perhaps worthwhile to first state the nature and goal of modeling in general. Modeling is not just putting forward mathematical equations; rather it should be seen as a scientific approach to understand the complexity of the problem at hand by

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making appropriate assumptions and simplifications (*1*, *2*). Thus, the first step is to describe the core of the problem. As a second step, this core information should be translated into mathematical equations (which makes it a mathematical model) and the third step is to validate the outcome of the calculations with experimental data. Often, this does not lead immediately to satisfactory results and an adjustment of the model may be needed, in other words modeling is of an iterative nature (*1*). If after some iterations the result is deemed satisfactorily, relevant kinetic parameters (such as rate constants, activation energy and entropy) can be estimated by regression techniques; this is done by fitting the model to the experimental data (*1*). After that, the final stage is reached, that is to say, future observations can be predicted, thereby partially reducing the need of new experiments, but only partially, because the predictions need to be checked. The better the predictions appear to be, the more trustworthy the model becomes.

Predictive models for foods are usually kinetic models, because most of the food problems have an element of time in them; food quality changes over time (usually deteriorates in time). With respect to kinetic models, a distinction can be made between mechanistic and empirical models (*1*). Mechanistic models are based on knowledge of the chemical reaction mechanism of the process under study. This is often in the form of differential equations based on the mass action law, stating that the rate of a reaction is proportional to the concentration of the reactants. The parameters in mechanistic models may be related to physical parameters such as rate constants, diffusion constants, partition constants and activation energies. Empirical models on the other hand provide a mathematical fit of the observations while the equations are not derived based on mechanistic insight. This will be necessary if it is, as yet, unknown what mechanism causes the observations. The parameters derived from empirical models do not have a physical interpretation, they just describe a mathematical fit. Incidentally, empirical models are not better or worse than mechanistic models, but with empirical models it is not well possible to come to the core. However, the power of empirical models is that they are able to describe a problem at hand without having to know all the details of the actual process.

Modeling of Flavor Compound Formation in the Maillard

Much of Maillard research is done with simplified model systems, which has obviously the big advantage that we come to the core relatively easy and that interpretation becomes more straightforward. The disadvantage however is that translation of the obtained results to real foods becomes problematic. On the other hand, if Maillard research is done with real foods, the results are often conflicting and quite uncertain, because there are so many uncontrolled confounding factors and as a result the models are of an empirical nature.

Compared to the modeling of color formation in the Maillard reaction, the number of papers on modeling of flavor compound formation is rather low. In general terms, it is fairly well established how the various Maillard flavor compounds are formed. Figure 1 shows a scheme derived from literature (*3*) in which this is depicted.

An example of an empirical model describing the kinetics of pyrazine formation in given in (*4*), where the authors applied a fractional first-order conversion model to describe their observations. In view of the complexity of pyrazine formation as shown in Figure 1, a first-order model is not justified from a mechanistic point of view, but it performed well as an empirical model. As an example of a mechanistic model, Jousse et al. (*5*) published an interesting paper on kinetic modeling of flavor compounds formed in the Maillard reaction. First, they simplified the general scheme as depicted in Figure 1 to a kinetic model with the essential rate determining steps. Their kinetic model is reproduced in Figure 2.

This is the first step mentioned in the introduction: defining the core of the model, and the kinetic model proposed in Figure 2 is typically a mechanistic one. Based on this core, Jousse et al. (*5*) proposed a mathematical model (differential equations for each step), but instead of estimating parameters themselves, they collected literature data to find the relevant parameter estimates, and they obtained surprisingly consistent results. They then made a real prediction by solving the differential equations numerically and confronted that with new experimental results (the validation step) focusing on Maillard flavor compounds such as pyrazines, carbonyls, furans, and pyroles. The model prediction for, e.g., pyrazines (Figure 3a) was in line with observations (not shown here; the original article also shows fits for other flavor compounds).

If model fits and/or model predictions are confronted with experimental determinations of one component (such as a pyrazine), this is called uniresponse modeling. However, the model depicted in Figure 2 not only allows prediction of flavor compounds but also of other intermediates (such as the Amadori compound, deoxyosones) and other end products (i.e, colored components). Figure 3b shows such a model prediction for some selected compounds. The differential equations derived from Figure 2 were solved numerically to make these predictions using the software package AthenaVisualStudio (www.athenavisual.com). This software allows so-called multiresponse modeling, i.e., one can compare simultaneously more than one component (*1*). Figure 3b shows that considerable formation of the Amadori compound is predicted, as well as extensive regeneration of the amino acid, and very little formation of melanoidins (the brown components). Although these compounds were not determined experimentally in the work of Jousse et al (*5*), it is known from other work (e.g., (*6–10*)) that the profiles shown in Figure 3b are not realistic because there is much too much Amadori product formed, too much amino acid regenerated, and too little of the melanoidins formed. The papers published so far on multiresponse modeling of the Maillard reaction (e.g., (*6–9*, *11*, *12*)) show that it is a very powerful tool to come to mechanistic kinetic models, where chemical and physical insights are merged with kinetics. A very recent paper has applied the concept to multiresponse modeling of flavor compound formation in the Maillard reaction (*13*).

While such insight is invaluable, it does not solve immediately the problem to make predictions for what happens in real foods. We propose that the use of chemical thermodynamics may be a next step in understanding what is happening in real foods, and such understanding may then subsequently be translated into kinetic models.

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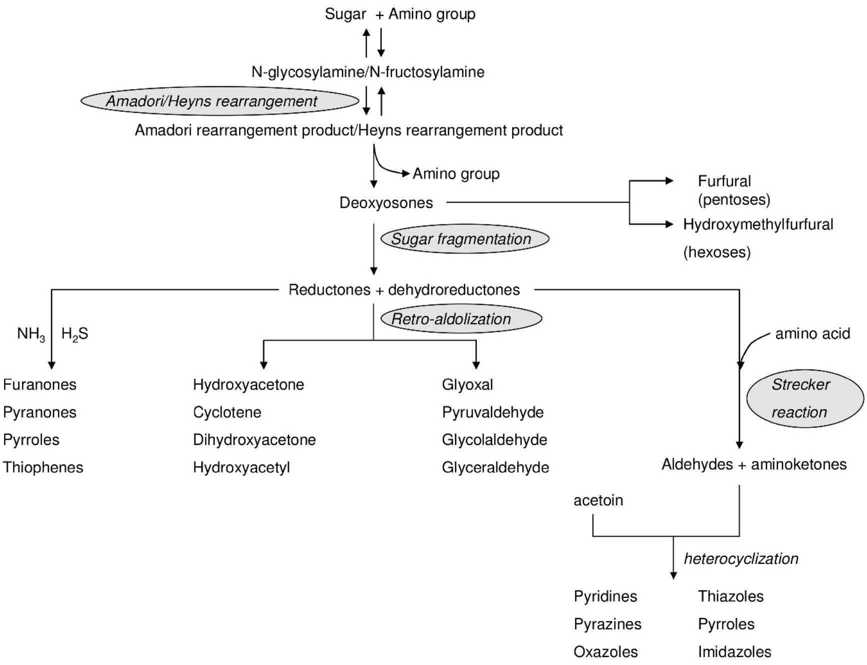
Sugar + Amino group
N-glycosylamine/N-fructosylamine
Amadori/Heyns rearrangement
Amadori rearrangement product/Heyns rearrangement product
Amino group
Deoxyosones
Furfural (pentoses)
Hydroxymethylfurfural (hexoses)
Sugar fragmentation
Reductones + dehydroreductones
Retro-aldolization
NH3
H2S
Furanones
Pyranones
Pyrroles
Thiophenes
Hydroxyacetone
Cyclotene
Dihydroxyacetone
Hydroxyacetyl
Glyoxal
Pyruvaldehyde
Glycolaldehyde
Glyceraldehyde
amino acid
Strecker reaction
Aldehydes + aminoketones
acetoin
heterocyclization
Pyridines
Pyrazines
Oxazoles
Thiazoles
Pyrroles
Imidazoles

Figure 1. Scheme of the Maillard reaction routes leading to formation of flavor compounds. Based on (3).

Chemical Thermodynamics as a Tool To Understand the Maillard Rreaction in Foods

The basic message from chemical thermodynamics is that a reaction only occurs if the free energy change of that reaction is negative. The present author has attempted to summarize the most basic elements of chemical thermodynamics for food scientists in much more detail (Chapter 3 in reference (*1*)). As a reminder, the free energy change ΔG (also called the Gibbs energy) has two components in it, namely enthalpy change ΔH and entropy change ΔS of a reaction:

$$\Delta G = \Delta H - T\Delta S \tag{1}$$

T represents absolute temperature. Free energy changes can be calculated for a proposed reaction from widely available thermodynamic data; this does not appear to have been done yet for the Maillard reaction, except for one compound in the Maillard reaction (*14*), but it could be a worthwhile, though tedious, exercise to do this more extensively. In any case, for chemical reactions, the concept of chemical potential has been introduced, which basically describes the molar free energy change. It can be derived that the chemical potential of a component i, μ_i, depends on its concentration c_i as follows:

$$\mu_i = \mu_i^{\ominus} + RT\ln\frac{c_i}{c^{\ominus}} \tag{2}$$

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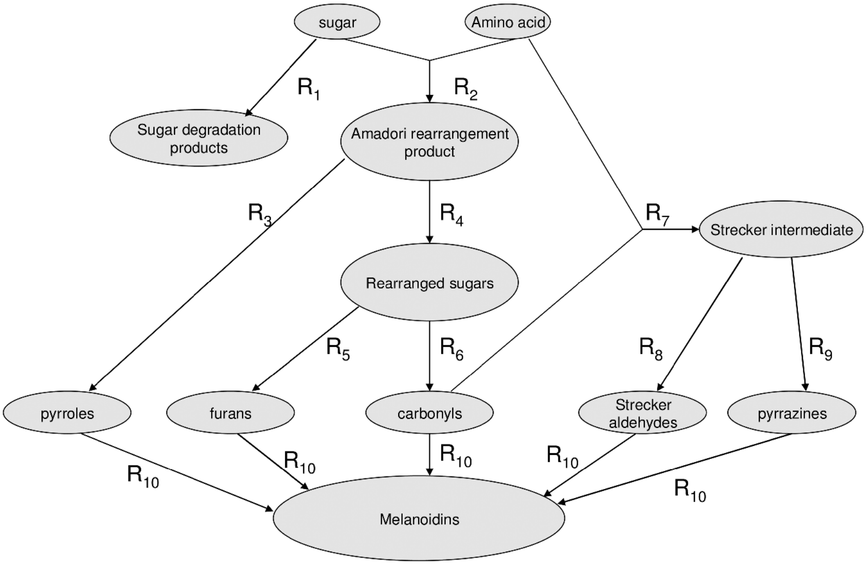
sugar
Amino acid
R_1
R_2
Sugar degradation products
Amadori rearrangement product
R_3
R_4
R_7
Strecker intermediate
Rearranged sugars
R_5
R_6
R_8
R_9
pyrroles
furans
carbonyls
Strecker aldehydes
pyrrazines
R_{10}
R_{10}
R_{10}
R_{10}
R_{10}
Melanoidins

Figure 2. Kinetic model for flavor compound formation in the Maillard reaction, where R_x indicates a rate determining step. Taken from (5).

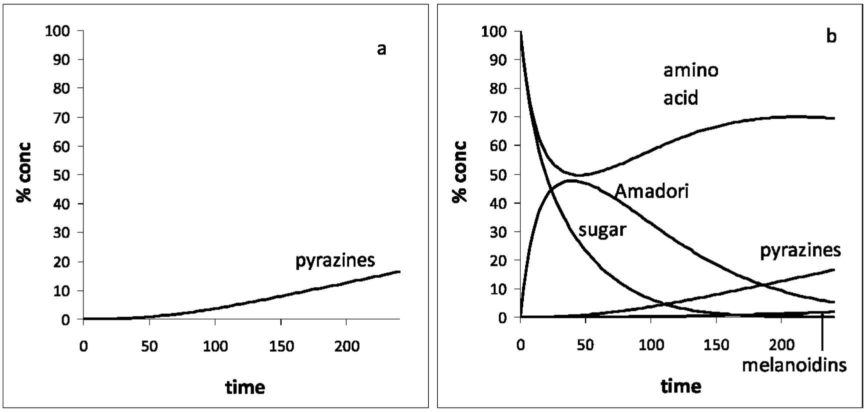

Figure 3. Uniresponse (a) and multiresponse (b) modeling of pyrazine formation by the kinetic model shown in Figure 2 and using kinetic parameters given in (5).

$\mu_i^{\ominus}$ is the standard chemical potential (i.e., at the standard concentration $c^{\ominus}$ = 1 mol dm^{-3} and 25 °C; $c^{\ominus}$ is necessary to make the concentration dimensionless), *R* is the gas constant. However, this is valid for ideal solutions only, i.e., for solutions in which on average the interaction between solute-solvent, solute-solute and solvent-solvent molecules are the same as in the pure liquids. Usually, solutions only behave ideally when they are very dilute. In real systems, including foods, a correction has to be made for interactions:

$$\mu_i = \mu_i^{\Theta} + RT \ln \frac{a_i}{a^{\Theta}} \qquad (3)$$

This equation introduces chemical activity a_i instead of concentration c_i (divided by the activity at standard conditions to ensure dimensionless units). The implications of this are large: we are used to deal with concentrations, i.e. number of molecules or particles per unit volume, but actually we should use their reactivity! Of course, there is a relation between activity and concentration:

$$a_i = y_i c_i \qquad (4)$$

y_i represents the molar activity coefficient of component i (instead of molar concentration one could also use the molality scale for which commonly the symbol γ is used to indicate the activity coefficient, or the mole fraction scale for which the symbol f is commonly used; most food scientists have learnt about water activity, which is usually expressed in the mole fraction scale, but the mole fraction scale for other components than water is not very practical for foods). The relation depicted in equation (4) is basically simple, but the problem is in finding values or expressions for the activity coefficient. For ionic components, the famous Debye-Hückel theory has been developed (incidentally, only valid for very dilute systems, but many variants are available that can also handle more concentrated solutions. Many empirical equations are available for activity coefficients, but unfortunately, very few for food science problems. See (*1*) for more details). In any case, the importance of the activity coefficient is that it tells us something about non-ideal behavior, and whether or not a correction has to be applied to the concentration. The numerical values of activity coefficients can be smaller or higher than unity but they cannot be negative. Cases are known where the activity differs orders of magnitude from the concentration (*1*), and so it is very worthwhile to know more about values of activity coefficients. As an example, Figure 4 shows activity coefficients of some (non-Maillard) volatile compounds in aqueous solutions.

Two effects can be noticed from Figure 4: i) the activity coefficients differ enormously from 1, ii) addition of sugar has a large effect on the activity coefficient. This latter effect is of importance in relation to foods, and it gives a hint on how important matrix effects can be: activity coefficients in foods will depend strongly on the composition of the food. While this is interesting, it adds to the complexity: will we ever be able to catch this complexity? Let's see how far we can get.

How does chemical thermodynamics relate to kinetics? Thermodynamics gives us a clue as to what is possible: namely only those reactions for which the free energy change is going to be negative, otherwise not (this applies to each reaction step, so a positive free energy change cannot be overcome by coupling it to a reaction that has a more negative free energy change, as is sometimes suggested in textbooks, this is simply not possible!). However, thermodynamics does not tell us how fast the possible will be reached, and this is where kinetics comes in. A striking example is the stability of a cube of sugar: according to thermodynamics this sugar is not stable and will eventually decompose into CO_2 and H_2O. However,

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it is everyone's experience that a sugar cube can be kept for a very long time without any noticeable change. The reason that thermodynamic equilibrium is not reached is that an energy barrier has to be overcome before that equilibrium can be reached and this is the realm of kinetics.

Without going into details, a connection can be made between kinetics and thermodynamics as follows (details can be found in Chapter 5 of reference (*1*)). Suppose we have a reaction between two reactants, A and B, leading to products P and Q.

$$\mathrm{A} + \mathrm{B} \rightarrow \mathrm{P} + \mathrm{Q} \tag{5}$$

According to the mass action law, the rate of formation of product P, for instance, is:

$$\frac{\mathrm{d[P]}}{\mathrm{d}t} = k_{\mathrm{obs}}[\mathrm{A}][\mathrm{B}] \tag{6}$$

k_{obs} represents the experimentally observed rate constant. We now connect this rate constant to theory, namely the transition state theory, in which an activated complex $\mathrm{AB}^{\ddagger}$ is postulated as an intermediate:

$$\mathrm{A} + \mathrm{B} \rightarrow \mathrm{AB}^{\ddagger} \rightarrow \mathrm{P} + \mathrm{Q} \tag{7}$$

Consequently, the rate expression in equation (6) now becomes:

$$\frac{\mathrm{d[P]}}{\mathrm{d}t} = k^{\ddagger}[\mathrm{AB}^{\ddagger}] \tag{8}$$

From statistical thermodynamics, it follows that:

$$k^{\ddagger} = \frac{k_{\mathrm{B}}T}{h} \tag{9}$$

in which k_{B} is Boltzmann's constant, and h Planck's constant. For thermodynamically ideal systems it can be derived that the rate constant k_{id} is:

$$k_{\mathrm{id}} = \frac{k_{\mathrm{B}}T}{h}\exp\left(-\frac{\Delta G^{\ddagger}}{RT}\right) \tag{10}$$

in which $\Delta G^{\ddagger}$ represents the free energy of activation. By combining several equations it follows that:

$$k_{\mathrm{obs}} = k_{\mathrm{id}}\frac{y_{\mathrm{A}}y_{\mathrm{B}}}{y^{\ddagger}} \tag{11}$$

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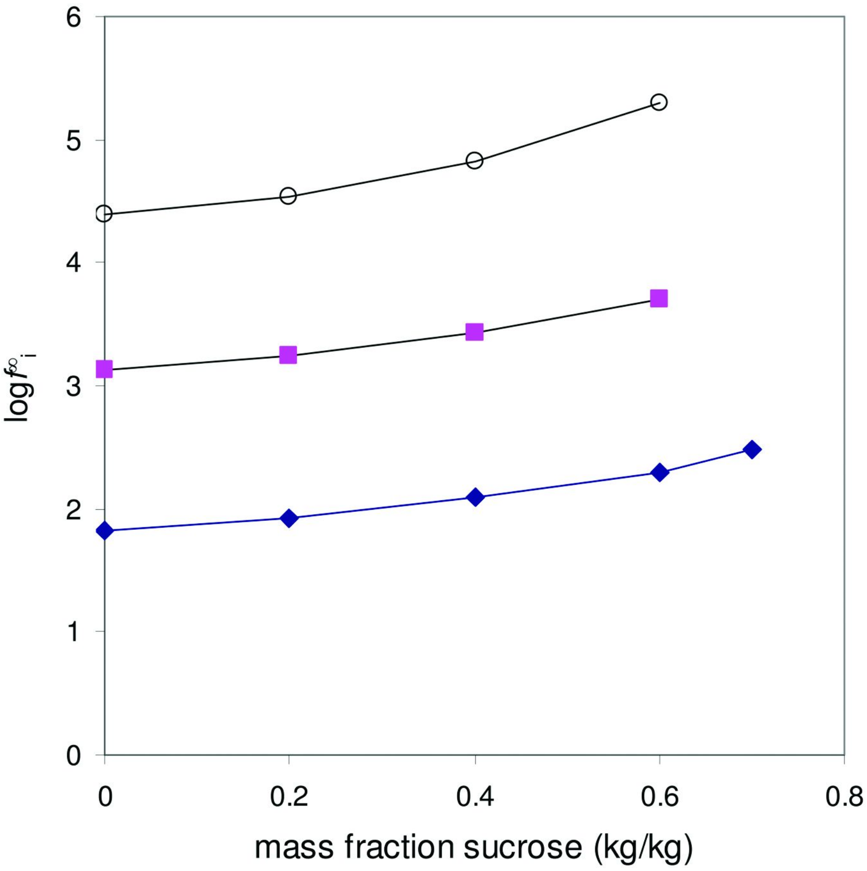

Figure 4. Activity coefficients for some volatiles in aqueous solution, ethylacetate (◆), butylacetate (■), n-hexylacetate (○), as a function of the amount of sucrose added. Data taken from (15).

This equation shows how the rate constant that applies to the ideal situation is modified by activity coefficients in non-ideal situations. It shows that if the ratio of the activity coefficients is unity, there is no effect of activity coefficients on the reaction rate, but if this ratio is > 1, then the rate constant will increase and if it is < 1, then it will decrease. As mentioned before, there is no direct way to predict activity coefficients from first principles, but experiments can give a clue. Although not in the field of Maillard chemistry, the following example shows that the effects can be large (Figure 5).

This example concerns the hydrolysis of an ester, and the graph shows that addition of sugars to the reaction mixture decreases the rate constant while addition of amino acids increases the rate constant of hydrolysis. It should be realized that the sugars and amino acids do not interfere with the actual elementary reaction, they just modify the activity coefficients of the reactants and of the activated complex, and exert an effect in that way. Looking at results as displayed in Figure 5, it is very likely that such effects play a role in foods and are one of the

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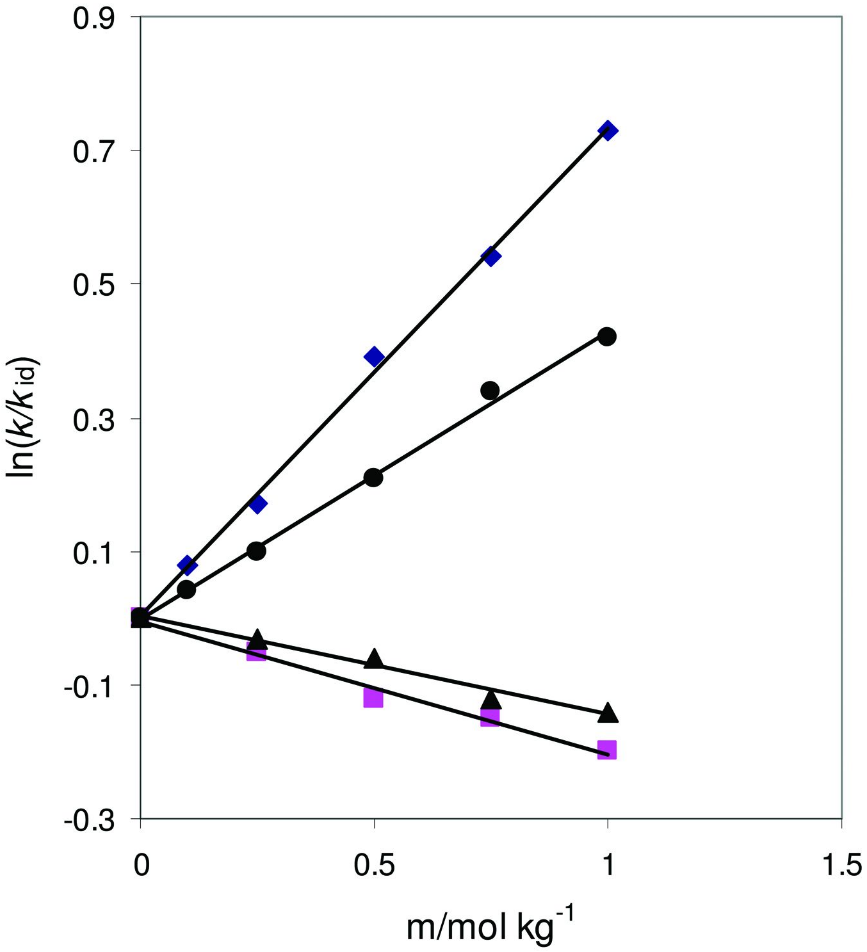

Figure 5. Effect of adding glucose (■), galactose (▲), glycine (◆), or alanine (●) on the rate of hydrolysis of 1-benzoyl-3-phenyl-1,2,4-triazole in an aqueous solution. Data taken from (15).

reasons why experiments with reactions in model solutions may deviate strongly from the same reactions in real foods.

Based on this, it is suggested that we now have a way to model food matrix effects quantitatively via activity coefficients and, if that view is accepted, the challenge then becomes to learn more about activity coefficients. Thus, it is suggested that we can get perhaps a better grip on matrix effects by paying more attention to thermodynamic effects. Admittedly, it will not be easy to find values for activity coefficients, but the few literature data available suggest that it is not impossible (e.g., (*16–18*)).

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Conclusion

The state of the art concerning kinetic modeling of flavor compound formation in the Maillard reaction has been discussed above. The chemical mechanisms that lead to flavor compound formation in the Maillard reaction are now fairly well established, and thus provide a basis for mechanistic models. Obviously, it is very useful to be able to quantitatively predict the amounts of flavor compounds that will be produced under certain conditions. In passing we note that such predictions do not tell anything about the perception of flavor compounds, that is a different subject. A conclusion about kinetic modeling is that adequate tools are available and that research in this direction can indeed lead to much more quantitative insight on the amounts of flavor compounds formed under specified conditions.

As for the problem on how to predict flavor compounds quantitatively in a food matrix, we have suggested that coupling of thermodynamics and kinetics may be the next step in moving forward to a better understanding of what is happening in real foods. Here, we are only at the beginning of understanding and it is hoped that more research will be initiated in this direction.

If we then summarize the points mentioned in a SWOT analysis, the following results emerge.

Strong points:

- Models allow a testable quantitative prediction of flavor compound formation based on mechanistic insight
- Models help in understanding the core of the problem by simplifying to the basics

Weak points:

- Translation from simplified model systems to real foods is problematic
- The very diverse and conflicting results in literature show the large uncertainty we are facing currently

Opportunities

- Modeling helps to come to a more unified approach that will reduce the uncertainty
- Coupling of kinetics and thermodynamics may help in getting a better and quantitative account of what is happening in the food matrix

Threats

- Food scientists seem to be somewhat uneasy with mathematical models
- Scientists focus too much on model systems rather than on reality

This SWOT analysis shows in a nutshell where we should focus on to come to a better understanding of flavor formation in the Maillard reaction. We conclude with two quotes that are very applicable to this problem. One quote is from Einstein who said: "*Models should be as simple as possible but no simpler than that*", illustrating the point that simplifications and assumptions are essential in a scientific endeavor but that they need to be critically checked. The other quote is from the economist Henry Theil who said: "*Models are to be used, not to be believed*", illustrating that models are a tool, no more and no less, to get a grip on reality.

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Chapter 2

Kinetic Modeling of the Formation of Volatile Compounds in Heated Beef Muscle Extracts Containing Added Ribose

Dimitrios P. Balagiannis,[1] Jack Howard,[1] Jane K. Parker,[1]
Neil Desforges,[2] and Donald S. Mottram*,[1]

[1]Department of Food and Nutritional Sciences, University of Reading,
Whiteknights, Reading RG6 6AP, United Kingdom
[2]Waltham Centre for Pet Nutrition, Waltham-on-the-Wold,
Melton Mowbray, Leicestershire LE14 4RS, United Kingdom
***d.s.mottram@reading.ac.uk**

Volatile compounds formed via the Maillard reaction contribute significantly to the flavor profile of heated foods. The reaction is dependent on many factors; among the most important are the type and concentration of the initial precursors. Changes in the precursor composition can result in considerable changes to the final volatile composition, thereby altering the organoleptic properties of the final product. In this study, the initial sugar concentration of an aqueous beef muscle extract was manipulated by adding sufficient ribose to increase the sugar content by 10-fold. The extract was heated at 130°C and the volatiles were analyzed by GC-MS at time intervals between 5 and 90 minutes. The effect of ribose concentration on the volatile profile was examined and the data were used to build a kinetic model that described the formation of ribose-derived volatile compounds.

Introduction

Thermal processing of food results in a cascade of reactions within the food matrix which leads to the generation of flavor compounds. These are mainly produced from the Maillard reaction, a series of parallel and consecutive sub-reactions, which are thermally induced and initiated by reaction between reducing

In Controlling Maillard Pathways To Generate Flavors; Mottram, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

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sugars and amino compounds present in the food. The chemistry of the formation of many flavor compounds has been well established, however, the quantitative control of flavor generation is difficult to accomplish, although it is desirable for industrial as well as for scientific purposes. It may be possible to achieve such control by elucidating the kinetics of Maillard reaction through modeling of its most important chemical pathways.

Multiresponse kinetic modeling is considered, mathematically and statistically, as a very appropriate method to use with such studies (*1*). The advantage of this method is that several responses are taken into account simultaneously and, as a result, improved estimates for model parameters and better mechanistic insight are achieved. Consequently, with this approach we can acquire the optimum information about the mechanism and the kinetics of a chemical reaction. A similar approach has already been used by several groups who have recently published models of the Maillard reaction (*2–12*). However, only a few are concerned with flavor chemistry and volatile formation.

Most kinetic studies on the Maillard reaction have been conducted with aqueous model systems consisting of simple mixtures of sugars and amino acids and/or proteins. In the present study, we have chosen to use a meat-like system, which resembles a real food system, in order to address the need for research in real foods. An aqueous extract of raw homogenized meat was used. This system comprised a mixture of sugars, amino acids, peptides, proteins and many other molecules, providing a complex reaction solution. To obtain better insight into the underlying chemistry and the competition between reactants, the effect of increasing the ribose concentration in the meat extract by 10-fold was studied.

Materials and Methods

Preparation and Cooking of Meat Extract

Beef muscle taken from sirloin steak was sliced, mixed with an equal quantity of deionized water and homogenized by blending it for 1 min using a domestic food processor (Megamix Cuisine Système 5100). The mixture was then centrifuged for 20 min at 14,000 rpm at 4°C and the supernatant was filtered (Whatman filter number 3) under vacuum. The ribose concentration was determined (1.16 mmol/kg) and the extract was split into two portions. D-Ribose was added to one portion to increase its concentration by 10-fold; the other portion was unchanged. Aliquots from both meat extracts (20 mL) were sealed in 30 mL glass ampoules, immersed in an oil bath at 130°C and heated for different time intervals from 5 to 90 min. At least two replicates were prepared for each heating time. After heating, each ampoule was immersed in a coolant at –50°C to stop the reaction.

Analysis of Volatiles

Dynamic headspace analysis was used for the extraction of the volatiles as described previously (*13*). Homogenized heated extract (5 g) and HPLC grade water (10 mL) were placed in a 250 mL conical flask with a Dreschel head and the volatiles were swept onto Tenax absorbent by nitrogen gas. The analysis of

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the components was performed on a Perkin-Elmer Clarus 500 GC-MS system (Perkin-Elmer, Beaconsfield, United Kingdom) coupled with an automated thermal desorption unit (Turbomatrix ATD, using a DB5 non-polar column (60 m × 0.32 mm i.d., 1 µm film thickness; J&W Scientific, Agilent) under instrument conditions described by Methven et al. (*13*).

A series of C_6-C_{25} n-alkanes were analyzed under the same conditions to obtain linear retention index (LRI) values for the components. The compounds were identified by comparing their mass spectra and LRI values with those for authentic compounds or published data or by comparison with spectra contained in the NIST/EPA/NIH Mass Spectral Database (MS Windows version 2.0a, 2002).

Quantification of the volatiles, which were used for modeling, was performed by generating calibration curves using the standard addition method. For all the other compounds, approximate quantification was obtained by comparison with the internal standard, as described by Methven et al. (*13*).

Analysis of Free Amino Acids and Sugars

Aliquots (1 g) of heated and unheated samples were mixed with 10 mL of hydrochloric acid (0.01 M) and stirred for 15 min at room temperature. The mixtures were then allowed to settle for 15 min, before an aliquot of the supernatant (1.5 mL) was removed, placed in a microtube and centrifuged at 7200 g for 15 min. It was then stored at –19°C until further analysis.

The free amino acids were measured using the EZ-Faast amino acid derivatisation technique (Phenomenex, Torrance, CA, USA). One hundred microliters of the centrifuged supernatant were derivatized and analyzed by GC-MS using an Agilent 5975 system (Agilent, Palo Alto, CA) in electron impact mode (*14*).

Sugar analyses were performed using a 8220i Dionex ion chromatography system (Dionex Corp., Sunnyvale, CA) (*14*). The extract was mixed with trehalose (internal standard) and then injected onto a Carbopac PA10 column (Dionex) using an autosampler. Two different solutions were prepared: A: H_2O, B: 400 mM NaOH. An isocratic program was employed for 30 min using 96% of solvent A and 4% of solvent B. For the next 10 min a solution of 40% of solvent A and 60% of solvent B was employed. Finally the column was re-equilibrated for another 10 min with 96% A and 4% B. A pulsed amperometric detector was used with the following settings: 420 ms at 0.05 V, 180 ms at 0.75 V, and 420 ms at -0.15 V, while the sensitivity was set at 3K. Chromatographic analysis of peaks was performed using Chromaleon (Dionex Corp., Sunnyvale, CA). Standards of glucose, fructose, sucrose, ribose, maltose and mannose were used for identification and quantification.

Modeling

Multiresponse modeling was performed using Athena Visual Studio software package (Athena Visual Software Inc., Naperville, IL; www.athenavisual.com).

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Results and Discussion

Flavor Precursors

The meat system that was used for the present study was found to contain four sugars: glucose, ribose, mannose and fructose. However, only the first two had a significant impact on the formation of volatile compounds since mannose and fructose were present in low concentration which did not change significantly during the heating period. In the raw, unadulterated meat extract, glucose had a higher concentration than ribose (Figure 1a). From the same figure it is clear that ribose is much more reactive than glucose, which is in agreement with the findings of other investigators (*15*, *16*). Even when the ribose was increased by a factor of ten, giving a higher initial concentration compared to glucose, virtually all the ribose reacted, whereas relatively high levels of glucose still remained after 90 min of heating.

The total concentration of free amino acids was calculated from the sum of 20 individual amino acids that were analyzed by GC-MS. Figure 2 shows that the initial slow decrease in total amino acid concentration was followed by a leveling off and/or a small increase. In general, the amino acids were present in the raw, un-adulterated extract, at higher molar concentrations than the sugars, and they were still present at relatively high concentrations at the end of the 90 min cook. This suggests that the sugar levels are the limiting factor in the Maillard reaction in meat, and not the levels of amino acids. In studies using simple model systems (*17–19*), it was also found that sugars react faster than the amino acids. Van Boekel (*20*) suggested that the changes in amino acid concentrations were the result of at least three reactions: the reaction with the sugar at the initial phase of the Maillard reaction, amino acid regeneration from Amadori products, and reactions with intermediate and advanced Maillard products. In the system used for this study, it is probable that proteolysis affected the amino acid concentration as well.

Effect of Ribose Addition on Volatile Profile

More than 100 compounds were identified from the volatile analysis. They included alcohols, unsaturated alcohols, aldehydes, unsaturated aldehydes, aromatic aldehydes, ketones, unsaturated ketones, cyclic ketones, pyrazines, thiazoles, pyrroles, dimethyl sulfides, furans and furanones.

In general, the concentration of lipid-derived compounds was not affected by the addition of ribose. For the Maillard compounds, the addition of ribose resulted in an increase in the final yield of volatiles. An exception was 2-ethyl-3,6-dimethylpyrazine where the addition of ribose caused a reduction in its concentration.

The most abundant compounds in the heated meat extract were the Strecker aldehydes 3-methylbutanal and 2-methylbutanal. From Figure 1(c,d) it can be seen that the effect of ribose addition resulted in different trends for the two compounds. For 2-methylbutanal, the concentration clearly increased during the whole heating period, and the concentration, at any given time, was consistently higher in the samples with the added ribose. On the other hand, 3-methylbutanal showed a different behavior: without added ribose, the shape of the curve

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was very similar to that of 2-methylbutanal, but the added ribose appeared to promote the formation of 3-methylbutanal much less than was observed for the 2-methylbutanal. After the first 40 min of heating, there was very little further accumulation of 3-methylbutanal, to such an extent that the final concentration of 3-methylbutanal was lower than in the extract with no added ribose. This leveling off is partly due to the depletion of sugars, but these curves represent the accumulation of the respective aldehydes, which is controlled by both formation and degradation pathways. Aldehydes like the methylbutanals can undergo further reaction as the Maillard reaction progresses. This was demonstrated in previous work on liver, which was cooked for a much longer period, where 3-methylbutanal levels reached a maximum and then started to decrease (*11*). In this study, degradation is particularly obvious for 3-methylbutanal in the extract with added ribose, where even during the early stages of the reaction, the accumulation of 3-methylbutanal is moderated by this degradation pathway and the same increase observed for the 2-methylbutanal when ribose was added was not achieved. Towards the end of the reaction, there was little change in the 3-methylbutanal levels since it was being degraded as fast as it was being formed. It is likely that 2-methylbutanal also degrades but the rate of its degradation to other products was slower than its formation rate. With a methyl substituent in the 2-position rather than the 3-position, 2-methylbutanal is likely to be less reactive both sterically and electronically towards further reactions, such as the aldol condensations.

Furfural is a compound which can be formed from both hexose and pentose sugars in a Maillard system. Because it contains five carbon atoms, dehydration of pentoses is the most common formation pathway cited by many investigators (*21–23*). However, hexose fragments that consist of five carbons may also give rise to furfural formation (*24*). In addition, from labeling studies of the caramelization reaction, it was proposed that furfural can be formed from the thermal decomposition of 5-hydroxy methylfurfural, which is a dehydration product from hexoses (*25*).

In the meat system under study, furfural was the furan compound with the highest concentration and was one of the most abundant volatile components. Figure 1b shows that the addition of ribose increased the final yield of furfural.

Modeling

In previous work (*11*), a kinetic model was obtained for the formation of 3-methylbutanal and 2-methylbutanal in a heated extract prepared from ox liver. In that extract, the predominant sugar was glucose and the proposed mechanism of the formation of these two volatile compounds is shown in Figure 3. It was assumed that glucose reacted with the amino compounds that were present in the system and, thence, a group of kinetically important intermediates (INT1) was formed. Probably, these intermediates were Amadori type products and/or their breakdown products such as deoxyhexosuloses (*26*). These reacted further to give a second group of intermediates (INT2), probably short chain dicarbonyl compounds.

Thus, in terms of a mechanistic model, it is appropriate to describe INT1 type intermediates as compounds that contain the whole carbon skeleton of their parent sugar in their molecule while the INT2 type compounds are shorter chain sugar breakdown products. The latter are considered very short-lived, because they react very fast, through diffusion controlled reactions, to other compounds. When INT2 interacts with leucine (Leu) and isoleucine (Ile), 3-methylbutanal and 2-methylbutanal are formed, respectively. When they react with all other free amino acids, amino acid-specific Maillard products are formed (M). This group includes Strecker compounds from other amino acids (such as 2-methylpropanal from valine or phenylacetaldehyde from phenylalanine) as well as melanoidins, which incorporate both free amino acids and INT2 into the molecule in a ratio of approx 1:1. Additional reactions of INT2 compounds, without the further involvement of amino acids, result in the formation of other Maillard type products (M′), such as protein and/or peptide bound products and condensation products with sugars, sugar fragments or more advanced stage Maillard reaction products.

As mentioned earlier, the major sugars found in the meat system used for the present study were ribose and glucose. Although the reaction scheme in Figure 3 can be used to model the glucose pathway, the group of intermediates initially formed from a pentose sugar is different to those formed from glucose. Consequently, it is necessary to update the mechanism of Figure 3 and to include ribose and its products as well. In a similar way to glucose, ribose-specific INT1 compounds ($INT1_{Rib}$) are formed from a reaction with rate constant k_5; then they react further through a reaction with rate constant k_6 and more compounds of the INT2 group of intermediates are formed (Figure 4).

As discussed above, several pathways have been proposed from the literature for furfural formation. Dehydration of pentose sugars is the most important when pentoses are present in a heated system but hexoses can also produce furfural. Consequently, furfural is derived from both $INT1_{Rib}$ and $INT1_{Glu}$ type compounds and as such it has been incorporated in the mechanism proposed for this study. Furthermore, it is known that furfural reacts further to give other compounds which contribute significantly to the color and flavor development within a Maillard system (*27–29*). This is shown in the present model with a pseudo first order reaction with a rate constant k_9 (Figure 4).

It is known that Strecker aldehydes can undergo condensation and other changes as they react further to give other compounds (*21*). Also, according to Blank et al. (*30*, *31*), the Strecker compounds formaldehyde and acetaldehyde can attach to pentose sugar derivatives (which are INT1 type compounds) to form 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (furaneol) and 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (homofuraneol), respectively. Similarly, it is valid to speculate that 3-methylbutanal and 2-methylbutanal participate in this type of reaction to give products analogous to furaneol and homofuraneol. In preliminary kinetic model processing, which included model selection and discrimination studies, 3-methylbutanal and 2-methylbutanal were found to give better models when their reaction to other products changed from first order to second order, with the participation of both ribose- and glucose-derived INT1 type intermediates. Furthermore, because it is also valid to assume that INT2 type compounds can also react with those two aldehydes, when we applied this to the model, it responded with undetermined estimates, which

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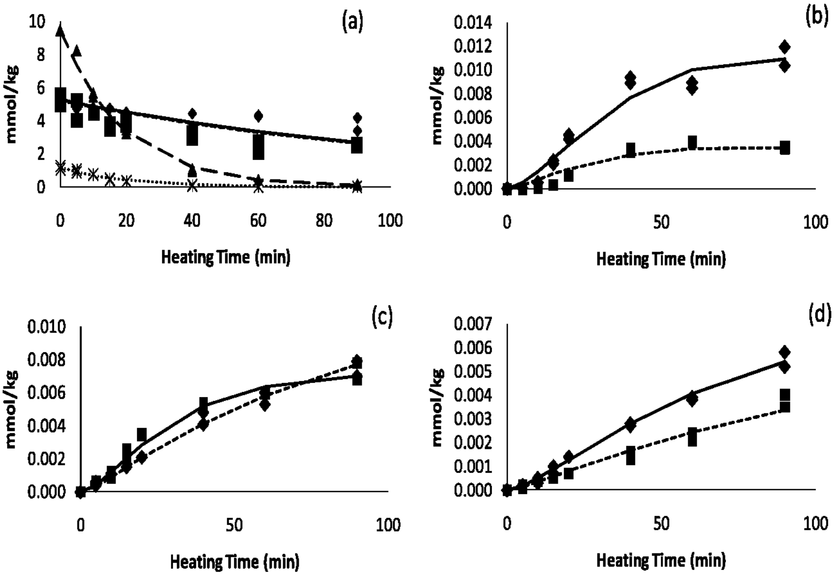

Figure 1. Experimental values and modeling predictions for precursors and volatile products in heated muscle extract with and without added ribose: (a) sugar precursors with (▲ ribose, ◆ glucose, — ribose model, ---- glucose model) and without (∗ ribose, ■ glucose, ···· ribose model, — glucose model) added ribose; (b) furfural with (◆ values, — model) and without (■ values, --- model) added ribose; (c) 3-methylbutanal with (■ values, — model) and without (◆ values, ···· model) with added ribose; (d) 2-methylbutanal with (◆ values, — model) and without (■ values, --- model) added ribose.

indicates that this chemical step was not kinetically important. Consequently, it is considered that INT1 are the kinetically important compounds which react with 3-methylbutanal and 2-methylbutanal to give other compounds.

The model comprises a number of differential equations that are derived directly from the mechanism of Figure 4 by applying the rate law theory for chemical reactions.

$$\frac{d[\mathrm{Glu}]}{dt} = -k_1[\mathrm{Glu}] \quad (1)$$

$$\frac{d[\mathrm{Rib}]}{dt} = -k_5[\mathrm{Rib}] \quad (2)$$

$$\begin{aligned}\frac{d[\mathrm{Int1_{Glu}}]}{dt} &= k_1[\mathrm{Glu}] - k_2[\mathrm{Int1_{Glu}}]R_{leu}F_{leu} - k_2[\mathrm{Int1_{Glu}}]R_{ile}F_{ile} - k_2[\mathrm{Int1_{Glu}}](1 - R_{leu}F_{leu} - R_{ile}F_{ile}) \\ &\quad - k_2[\mathrm{Int1_{Glu}}] - k_3[\mathrm{3MeBut}][\mathrm{Int1_{Glu}}] - k_4[\mathrm{2MeBut}][\mathrm{Int1_{Glu}}] - k_7[\mathrm{Int1_{Glu}}]\end{aligned} \quad (3)$$

$$\begin{aligned}\frac{d[\mathrm{Int1_{Rib}}]}{dt} &= k_5[\mathrm{Rib}] - k_6[\mathrm{Int1_{Rib}}]R_{leu}F_{leu} - k_6[\mathrm{Int1_{Rib}}]R_{ile}F_{ile} - k_6[\mathrm{Int1_{Rib}}](1 - R_{leu}F_{leu} - R_{ile}F_{ile}) \\ &\quad - k_6[\mathrm{Int1_{Rib}}] - k_3[\mathrm{3MeBut}][\mathrm{Int1_{Rib}}] - k_4[\mathrm{2MeBut}][\mathrm{Int1_{Rib}}] - k_8[\mathrm{Int1_{Rib}}]\end{aligned} \quad (4)$$

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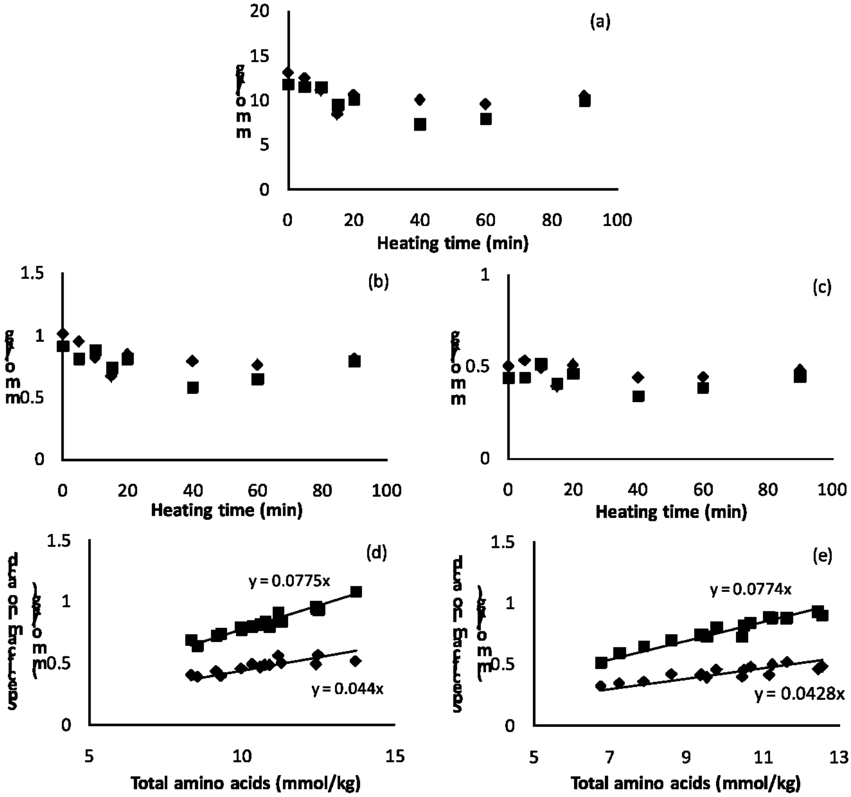

Figure 2. Changes in the concentration of (a) total free amino acids, (b) leucine and (c) isoleucine with (◆) and without (■) added ribose, during heating of meat extract at 130°C. Relationship between the concentrations leucine (■) and isoleucine (◆) and the total free amino acid for the samples with (e) and without (d) added ribose.

$$\frac{d[\mathrm{3MeBut}]}{dt} = k_2[\mathrm{Int1_{Glu}}]\mathrm{R_{leu}F_{leu}} + k_6[\mathrm{Int1_{Rib}}]\mathrm{R_{leu}F_{leu}} - k_3[\mathrm{3MeBut}]([\mathrm{Int1_{Glu}}] + [\mathrm{Int1_{Rib}}]) \qquad (5)$$

$$\frac{d[\mathrm{2MeBut}]}{dt} = k_2[\mathrm{Int1_{Glu}}]\mathrm{R_{ile}F_{ile}} + k_6[\mathrm{Int1_{Rib}}]\mathrm{R_{ile}F_{ile}} - k_4[\mathrm{2MeBut}]([\mathrm{Int1_{Glu}}] + [\mathrm{Int1_{Rib}}]) \qquad (6)$$

$$\frac{d[\mathrm{M}]}{dt} = k_2[\mathrm{Int1_{Glu}}](1 - \mathrm{R_{leu}F_{leu}} - \mathrm{R_{ile}F_{ile}}) + k_6[\mathrm{Int1_{Rib}}](1 - \mathrm{R_{leu}F_{leu}} - \mathrm{R_{ile}F_{ile}}) \qquad (7)$$

$$\frac{d[\mathrm{M'}]}{dt} = k_2[\mathrm{Int1_{Glu}}] + k_6[\mathrm{Int1_{Rib}}] \qquad (8)$$

$$\frac{d[\mathrm{Furf}]}{dt} = k_7[\mathrm{Int1_{Glu}}] + k_8[\mathrm{Int1_{Rib}}] - k_9[\mathrm{Furf}] \qquad (9)$$

Equations 1−3, 8 and 9 derive directly from the proposed mechanism while equations 4567 are more complicated because they contain the parameters $\mathrm{R_{leu}}$, $\mathrm{R_{ile}}$, $\mathrm{F_{leu}}$ and $\mathrm{F_{ile}}$. As was mentioned previously, 3-methylbutanal, 2-methylbutanal, M and M′ are products of diffusion controlled reactions since INT2 type intermediates are short lived and very reactive. Consequently, it is actually the INT1 concentration, which is controlled by the rate constants k_1, k_2, k_5 and k_6 (Figure 4), that limits the formation of those compounds. Furthermore, 3-methylbutanal and 2-methylbutanal are formed in proportion to the ratio of the parent amino acids to the total amino acid concentration that is present in the system under study (*11*) and the constants R_{leu} and R_{ile} express these ratios for leucine and isoleucine respectively. As shown in Figure 2(d,e), these were determined to be 0.0778 and 0.0425 for the extract with no added ribose and 0.0774 and 0.0432 for the extract with added ribose, respectively. It is encouraging that the ratios for the two amino acids have almost the same values for the two types of extract. The parameters F_{leu} and F_{ile} express the ratio of leucine and isoleucine that were converted to the corresponding methylbutanals. Finally, the factor $1- R_{leu} *F_{leu}-R_{ile} *F_{ile}$ defines the ratio of the concentration of all the amino acids present in the studied system, except the leucine and isoleucine, to the total amino acid concentration

Using the data that were collected from the analysis of the samples with the Athena Visual Studio software, the optimal estimates for the parameters k_1, k_2, k_3, k_4, k_5, k_6 ,k_7, k_8, k_9, R_{leu}, R_{ile}, F_{leu} and F_{ile} were estimated along with their 95% highest posterior density (HPD) intervals, which is a measure of their variability (Table I). In general, the HPD intervals have relatively low values except for k_2 and k_7. Also, as can be seen from Figure 1, the fit of the model to the experimental data is satisfactory. Only glucose presented a less acceptable fit and this is the reason that the HPD intervals for k_2 and k_7 were high.

For the estimated values, it can be seen that k_5 is higher than k_1, a result which was expected since it is well known that ribose is more reactive than glucose. Also, k_6 is lower than k_2 which means that the ribose-related intermediates $INT1_{rib}$ are less reactive than the corresponding glucose related intermediates and accumulate. This agrees with Hofmann's study (*32*), which found that glucose-related sugar fragments had higher concentrations than the corresponding fragments of xylose when they were individually refluxed, with an equimolar amount of L-alanine. Furthermore, k_3 is higher than k_4 which means that 3-methylbutanal degrades faster than 2-methylbutanal. This explains why the leveling off of these aldehydes is more apparent in Figure 1c (3-methylbutanal) than in Figure 1d (2-methylbutanal), especially with the higher level of ribose. Finally, according to the parameter estimation, k_7 is higher than k_8. This result suggests that $INT1_{glu}$ intermediates react faster to produce furfural than the corresponding $INT1_{rib}$ compounds and despite the higher value of k5 cf. k1, this model predicts that more furfural is derived from glucose than ribose. This is an interesting estimation but needs further investigation, since it was expected that furfural would be formed faster from ribose and ribose-related intermediates than from glucose.

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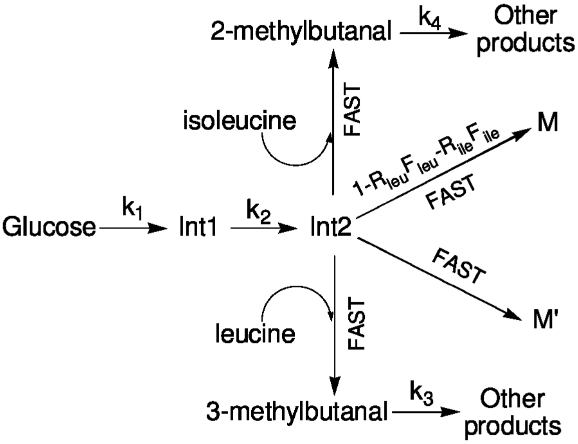
2-methylbutanal
k4
Other products
isoleucine
FAST
1-R_leu F_leu -R_ile F_ile
FAST
M
Glucose
k1
Int1
k2
Int2
FAST
M'
leucine
FAST
3-methylbutanal
k3
Other products

Figure 3. Mechanism of the formation of 3-methylbutanal and 2-methylbutanal in heated liver extract. (Reproduced from reference (11))

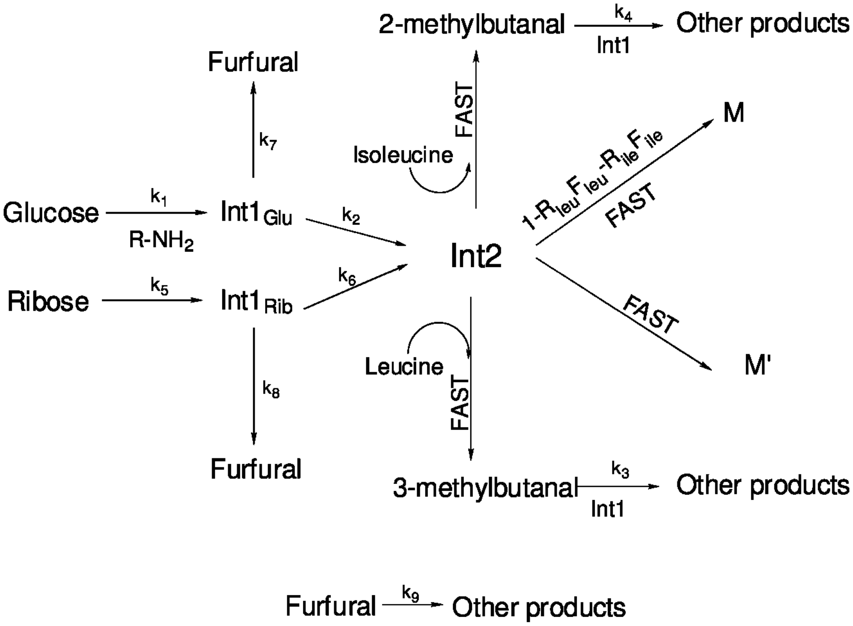
2-methylbutanal
k4
Int1
Other products
Furfural
k7
FAST
Isoleucine
1-R_leu F_leu -R_ile F_ile
FAST
M
Glucose
k1
R-NH2
Int1 Glu
k2
Int2
Ribose
k5
Int1 Rib
k6
FAST
M'
k8
Leucine
FAST
Furfural
3-methylbutanal
k3
Int1
Other products
Furfural
k9
Other products

Figure 4. Mechanism of the formation of 3-methylbutanal, 2-methylbutanal and furfural in heated muscle extract

By comparison of the values that were obtained from this model with the values that had been published previously for the liver extract model (Figure 3) (*11*), it seems that the parameters are of the same order of magnitude. Thus, it is possible that, with the analysis and incorporation in the model of a few more key compounds, such as the total amino compounds present in the system and/or compounds which belong to the first group of intermediates (e.g. Amadori products

Table I. Optimal estimates and 95% Higher Posterior Density intervals for the parameters which comprise the model of Figure 4

Parameter	*Optimal Estimates*
k1 (min^{-1})	7.52E-03 ± 1.68E-03 (22%)
k2 (min^{-1})	1.68E-01 ± 1.29E-01 (77%)
k3 (mmol kg^{-1} min^{-1})	4.28E-03 ± 1.17E-03 (27%)
k4 (mmol kg^{-1} min^{-1})	1.16E-03 ± 6.09E-04 (53%)
k5 (min^{-1})	5.20E-02 ± 2.73E-03 (5%)
k6 (min^{-1})	3.50E-03 ± 1.06E-03 (30%)
k7 (min^{-1})	9.39E-04 ± 7.65E-04 (81%)
k8 (min^{-1})	4.16E-05 ± 9.68E-06 (23%)
k9 (min^{-1})	2.73E-02 ± 8.58E-03 (31%)
F_{leu}	7.89E-02 ± 1.37E-02 (17%)
F_{ile}	5.56E-02 ± 9.87E-03 (18%)

and deoxyosones), a better model, common for both liver and sirloin meat types, might be achieved.

Limitations of the Model

The aim of the model presented was to simulate the formation of some important volatile compounds; this was achieved by combining a mechanism derived from literature data with experimental results. It has been stated that *"all models are wrong but some are useful"* (George Box cited in (*33*)), and the present model does have some limitations. Concerning the pseudo first order reaction of the initial step of the model (Figure 4 and equations 1 and 2), the exact concentration and behavior of the amino compounds present in the system is unknown, and it was assumed to be in excess during the whole cooking period. Additionally, the compounds INT1 and INT2 have not been quantified and, in particular, the information that could be obtained from the analysis of INT1 type intermediates, which are kinetically important compounds, would be valuable for the evolution and accuracy of the model. In addition, the high HPD intervals for k_2 and k_7 revealed the need for manipulation of glucose concentration in order to obtain, on the one hand, better estimates for those glucose-related parameters and, on the other hand, more insight into the mechanism. Thus, it would be important to conduct additional experiments in order to achieve a more robust and accurate model; in fact, this need reflects the iterative nature of modeling. Nevertheless, this study offers useful information about the underlying Maillard chemistry and it also supports the usefulness of multiresponse kinetic modeling in real food systems.

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Chapter 3

Dicarbonyl Intermediates: A Control Factor in the Maillard Reaction

Yu Wang[*] and Chi-Tang Ho

**Department of Food Science, Rutgers University, 65 Dudley Road,
New Brunswick, NJ 08901-8520
[*]ywang11@MIT.edu**

Maillard reaction starts from two simple reactants, the reducing sugar and amino acid, but it is a very complicated reaction consisting of many parallel and consecutive reaction cascades. Each of those reaction cascades, in which the corresponding intermediate (e.g. 3-deoxyosone, methylglyoxal or formaldehyde) are involved, would lead to the generation of a group of specific flavor compounds. All these intermediates occur in the Maillard reaction, and participate in different reaction cascades competitively. How the reaction goes depends on the competitiveness of each intermediate. Controlling the Maillard reaction is indeed to control the kinetic and mechanisms of intermediate formation. First of all, types of reactants and reaction conditions (temperature ranges, pH and water activity) definitely influence the generation of intermediates. Second, adding trapping agents of intermediates is considered as an effective way to block the reaction cascade and change the Maillard reaction.

Introduction

Dicarbonyl intermediates such as glyoxal (GO) or methylglyoxal (MG), which can be generated from Schiff's base and Amadori compounds in Maillard reaction, play important roles as precursors of aroma and color compounds especially in the Strecker degradation, a major flavor generation reaction. Dicarbonyl compounds with amino acids undergo Schiff's base formation,

In Controlling Maillard Pathways To Generate Flavors; Mottram, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

decarboxylation and α-aminoketone condensation leading to heterocyclic aroma compounds such as pyrazines, pyrroles and pyridines.

As mentioned above, dicarbonyl intermeduates could lead to some important flavor generation in Maillard reaction, which has been extensively studied for almost a century, particularly for the development of flavor and color in processed foods. Maillard reaction starts from very simple reactants, a carbonyl group (i.e., reducing sugar) and an amine group (i.e., amino acids), and goes through initial, intermediate and final stages in generation of Maillard products. Formation of those Maillard compounds (i.e., dicarbonyl compounds or flavor compounds) depends on the types of sugars and amino acids as well as reaction temperature, time, pH and water content which may alter reaction kinetics and pathways. For example, fructose shows a higher reactivity in 5-hydroxymethyl-2-furfural (HMF) generation compared to glucose and sucrose (*1*, *2*), and the formation pathways of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF) vary with different sugars and water content (*3*, *4*).

Furanoids, incorporating a furan ring into its molecular structure such as furfural, 2-acetylfuran, DMHF and HMF, are important flavor or flavor intermediates in foods. Most furanoids are formed from dicarbonyl intermediates in the presence or absence of amino acids during intermediate or final stage of Maillard reaction. HMF or furfural and DMHF are products obtained on the decomposition of 3-deoxyosone and 1-deoxyosone, respectively (*5*). These furanoid compounds, on one hand, can be formed through cyclization of an intact deoxyosone. On the other hand, the deoxyosone may be cleaved into small fragments such as MG or 1-hydroxy-2-propanone, which may recombine to form furanoids. Depending upon the reaction conditions, particularly the type of sugars and amino acids, the formation pathways for furanoid compounds may become more complicated. However, systematic studies of furanoid formation have not been performed yet. Furanoids particularly DMHF and 2-acetylfuran were chosen as the targets, and studied on the relationship between their generation and dicarbonyl intermediates formation, influence of types of amino acids or sugars. We have conducted two different experiments, DMHF generation through MG, and amino acid-dependent formation pathways of 2-acetylfuran and DMHF to give an overview of controlling Maillard reaction through dicarbonyl intermediates.

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DMHF Formation from MG

DMHF as an intense caramel-like aroma compound with low odor threshold has been widely used in the flavor industry. So far, the studies on formation pathways of DMHF have been mainly focused on different hexoses, 6-deoxysugars and pentoses. In the present study, the generation of DMHF from MG, an important flavor precursor for processed foods was carried out. Generally, MG and 1-hydroxy-2-propanone, two of major degradation products in the Maillard Reaction, could react together to form DMHF via 2,5-dioxo-3,4-dihydroxyhexane (*8*). However, when MG was heated alone at 120°C for 1 h, the formation of DMHF was observed (Figure 1), and the DMHF level was significantly increased as pH of the reaction increased. MG, one of the dicarbonyl compounds, may transform into 1-hydroxy-2-propanone through the Cannizzaro reaction (Figure 2), and subsequently lead to DMHF by reacting MG with 1-hydroxy-2-propanone (*6*). DMHF formation from MG was pH-dependent because the Cannizzaro reaction is a base catalyzed reaction.

The aroma compounds generated in the Maillard reaction depend on the composition of sugars, amino acids as well as the reaction condition. DMHF was produced at different levels either directly from MG or from MG in the presence of glycine or cysteine. At pH 8, Strecker degradation was the major reaction which consumed most of MG in the presence of amino acids. However, cysteine could be degraded into hydrogen sulfide which can be used as a reducing agent to produce 1-hydroxy-2-propanone from MG. At pH 5, Cannizzaro reaction and Strecker degradation became weaker, and reduction activity of cysteine was the main effect on DMHF formation, consequently cysteine reacting with MG generated a high level of DMHF. Cysteine may change its role from a reductant to an inhibitor at pH 3.

Maillard reaction involving thermal degradation of carbohydrates and amines could induce a complex reaction cascade in which aroma, taste and color compounds are generated through cyclization and fragmentation of carbohydrates. DMHF, on one hand, can be formed through cyclization of an intact carbohydrate. On the other hand, the carbohydrate may be cleaved into fragments such as MG and 1-hydroxy-2-propanone, which may recombine to form DMHF. In order to show DMHF formation mechanisms, the carbon module labeling (CAMOLA) was used. CAMOLA is a powerful technique to elucidate different pathways and evaluate the relative importance of each pathway (*6*). Equal molar of [^{13}C6] labeled and [^{12}C6] unlabeled glucose were mixed in the presence of certain amino acid and heated at 145°C for 1 hour, and the isotopomers of DMHF were analyzed by GC-MS. If the glucose carbon skeleton keeps intact in DMHF formation, equal molar of [^{13}C6] labeled DMHF and [^{12}C6] unlabeled DMHF should be obtained. However, if the fragmentation of glucose occurs before DMHF formation, up to seven isotopomers with different numbers of labeled carbons may be formed.

The results demonstrated that a 1:1 mixture of [^{13}C6]DMHF and [^{12}C6]DMHF was obtained (Figure 3A) in the presence of glycine or cysteine, suggesting no breakdown of glucose during DMHF formation. As an important intermediate during thermal degradation of glucose, MG itself could generate DMHF with or without amino acids. If a 1:1 mixture of [^{13}C6]glucose and [^{12}C3]MG reacted with glycine or cysteine at 145°C, a 4:1 mixture of [^{13}C6]DMHF and [^{12}C6]DMHF was obtained. Only 20% of DMHF was formed from MG, and the rest 80% was formed from glucose (Figure 3B).

In all of DMHF isotopomers, only [^{13}C6] labeled and [^{12}C6] unlabeled, were observed indicating that glucose kept carbon skeleton intact during DMHF formation in the presence of glycine or cysteine even if its fragment MG was added into system. However, in the presence of proline, [^{13}C3]DMHF became the major isotopomer, and about 60% of DMHF was formed from the cleavage of glucose and recombination (Figure 4A).

If green tea was added into the system, a significant decrease of [^{13}C3]DMHF isotopomer was observed suggesting that polyphenol compounds in the green tea

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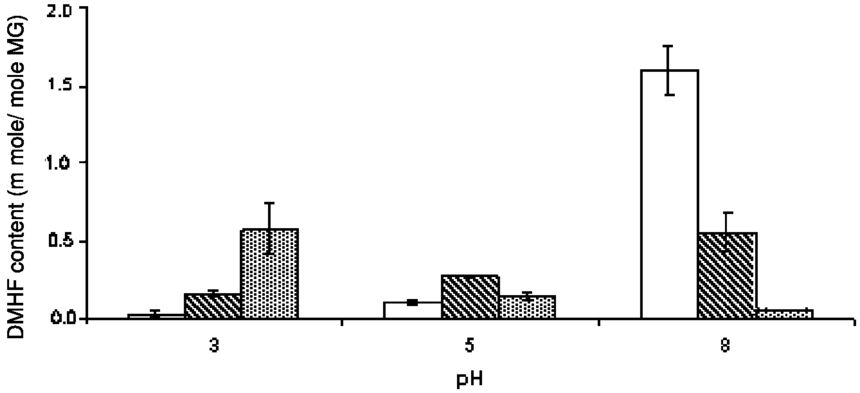

Figure 1. Effect of pH on 2,5-dimethyl-4-hydroxy-3(2H)-furanone formation in 1.4 M MG phosphate buffer solution incubated with or without 1.0 M glycine or 1.0 M cysteine: MG only (open bars); MG-cysteine (slash bars); MG-glycine (dotted bars). Values for DMHF are the means ± standard deviation (SD), each analyzed three independent times. Statistical significance was examined using Student's t-test comparison between the means. A p value of <0.05 was considered statistically significant.

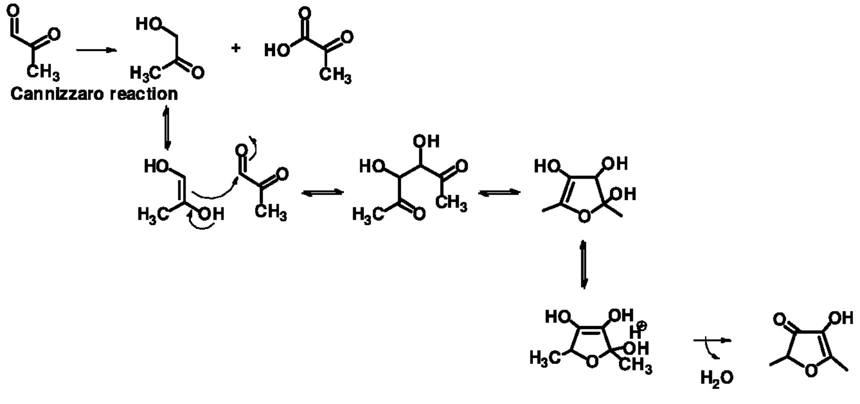

Figure 2. Reaction pathway leading methylglyoxal to 2,5-dimethyl-4-hydroxy-3(2H)-furanone via Cannizzaro reaction.

could trap dicarbonyl intermediates (Figure 4B). Therefore, trapping agent could be used to change the formation pathway from the recombination of fragments to the cyclization of intact carbon skeleton.

Amino Acid-Dependent Formation Pathways of 2-Acetylfuran and DMHF

Tables I and II demonstrate the labeling distribution of 2-acetylfuran and DMHF in different amino acid models with equal molars of [^{13}C6] labeled and [^{12}C6] unlabeled glucose. For the DMHF formation, compared to glycine

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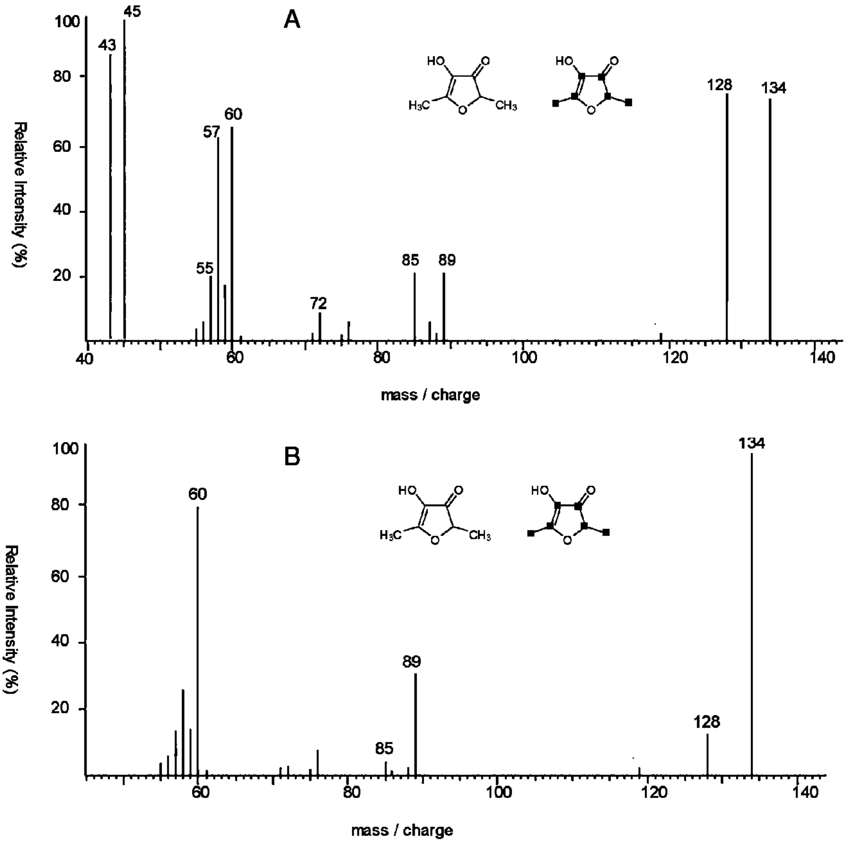

Figure 3. GC-MS spectrum of 2,5-dimethyl-4-hydroxy-3(2H)-furanone from a 1:1 mixture of [^{13}C6]glucose and [^{12}C6]glucose (A), and [^{13}C6]glucose and [^{12}C3]methylglyoxal (B) in the presence of glycine or cysteine

or cysteine, in the presence of proline, 60% of DMHF was formed from the cleavage of glucose indicating that type of amino acids would influence the reaction pathways. The formation of 2-acetylfuran showed a different behavior from DMHF in the glycine system. Firstly, a 1:1 mixture of [^{13}C6] 2-acetylfuran and [^{12}C6] 2-acetylfuran was observed in the model of cysteine suggesting no fragmentation. Secondly, model of proline showed about 25% of [^{13}C6] 2-acetylfuran and [^{12}C6] 2-acetylfuran, and the other half of 2-acetylfuran were due to fragmentation. Thirdly, glycine revealed a different characteristic from other amino acids in 2-acetylfuran formation. [^{12}C6] isotopomer showed in a higher level as compared to other [^{13}C6] isotopomer. The major fragmentation product was the [^{13}C5] 2-acetylfuran, while the percentage of its corresponding isotopomer [^{13}C1] was zero. Moreover, the [^{12}C6] isotopomer showed a higher level than [^{13}C6] isotopomer. Therefore, based on the principals of CAMOLA, the glycine may take part in the reaction causing a significant difference in the distribution of corresponding isotopomers.

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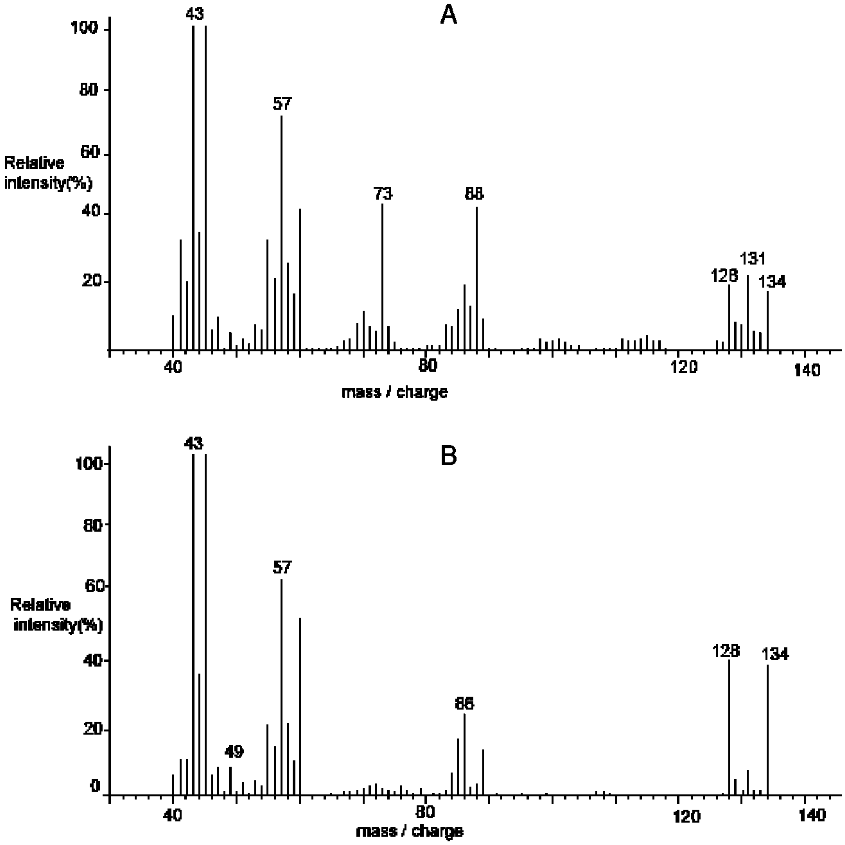

Figure 4. GC-MS spectrum of 2,5-dimethyl-4-hydroxy-3(2H)-furanone from a 1:1 mixture of [^{13}C6]glucose and [^{12}C6]glucose in the presence of proline (A) and in the presence of proline and green tea extract (B).

Table I. Percent Labelling Distribution of 2-Acetylfuran

Sample	*M*	*M+1*	*M+2*	*M+3*	*M+4*	*M+5*	*M+6*
Glc(C12/C13)+Cys	50	0	0	0	0	0	50
Glc(C12/C13)+Pro	24	9	5	25	6	9	22
Glc(C12/C13)+Gly	44	0	0	9	0	30	17

Table II. Percent Labelling Distribution of DMHF

Sample	*M*	*M+1*	*M+2*	*M+3*	*M+4*	*M+5*	*M+6*
Glc(C12/C13)+Cys	50	0	0	0	0	0	50
Glc(C12/C13)+Pro	16	2	2	60	2	2	16
Glc(C12/C13)+Gly	50	0	0	0	0	0	50

In order to confirm this interpretation, [2-^{13}C]-L-glycine was reacted with [^{12}C6] glucose and the result indicated that half of 2-acetylfuran was from recombination of glucose and glycine fragments. Generally, flavor compounds can be generated either from sugars or from sugars and amino acids. In the presence of other amino acids, only glucose contributed to the carbons of 2-acetylfuran, whereas in the presence of glycine, one carbon of 2-acetylfuran was from glycine. Glycine can be degraded into formaldehyde via Strecker degradation. Further fragmentation of the 1-deoxyosone may lead to the five carbons aldehyde which could react with formaldehyde via enolization and aldol condensation.

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Conclusion

Flavor compound formation in the Maillard reaction depends on various factors, such as the type of sugars and amino acids, reaction temperature, time, pH and water content. Generally, sugar and amino acid type may influence the flavor type, yield and formation pathway. Based on selected amino acids, 2-acetylfuran and DMHF demonstrated different formation pathways. For the DMHF, in the presence of cysteine, glucose carbon skeleton kept intact, while in the presence of proline, DMHF was formed from the recombination of glucose fragments. However, 2-acetylfuran could be formed either from glucose or from glucose and glycine. In the presence of glycine, [C-5] unit of glucose combined with formaldehyde from glycine leading to 2-acetylfuran. For other amino acids, either cyclization of intact glucose or recombination of glucose fragments could lead to 2-acetylfuran formation.

The type of reactant and reaction conditions are just some aspects in controlling Maillard reaction. If all these factors are fixed, for example, in our glucose and glycine model, glucose can be degraded into [C_1-C_5] fragments, and glycine can give rise to formaldehyde. All these molecules occur in the Maillard reaction and participate competitively in different reaction cascades. How the reaction proceeds depends on the competitive ability of each molecule. Therefore, although both DMHF and 2-acetylfuran contain a furan ring and can be formed from deoxyosone, in the glucose-glycine Maillard reaction DMHF generated only from cyclization of intact glucose, whereas 2-acetylfuran formed either from intact glucose or from [C_5] fragment and formaldehyde. Therefore, the carbonyl intermediate is the control factor in the controlling of Maillard reaction, and inhibiting or preventing their generation gave a rise to directing desired pathways of Maillard reaction.

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Chapter 4

Dicarbonyls from Maillard Degradation of Glucose and Maltose

M. A. Glomb,* J. Gobert, and M. Voigt

Martin-Luther-University Halle-Wittenberg, Institute of Chemistry,
Food Chemistry and Environmental Chemistry, Kurt-Mothes-Str. 2,
06120 Halle, Germany
***marcus.glomb@chemie.uni-halle.de**

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A thorough study on the formation of α-dicarbonyl compounds from lysine-glucose/maltose Maillard reaction mixtures is presented. Dicarbonyls were trapped by *o*-phenylenediamine to yield stable quinoxalines. Isolation of authentic references was done by multilayer countercurrent chromatography. Incubations of independent synthesized dicarbonyls and of stable isotope labeled sugars, and depletion from oxygen allowed insights into the mechanistic relationship of dicarbonyls detected. Within the Maillard-chemistry of glucose 1-deoxyglucosone for de-aerated and glucosone for aerated systems were identified as the central intermediates leading to C4- and C5-fragments, respectively. In maltose reactions 1-lysinyl-1,4-dideoxy-2,3-hexodiulose was of major importance.

From the beginning, Maillard reactions have been associated with the formation of flavor, taste and color in thermally processed foods (*1*). Reductone structures can prolong shelf life during storage. Protein modifications lead to a change in value of nutrition, but also of structure, which is another important aspect in consumer acceptance. Negative aspects include the formation of mutagenic and carcinogenic products. It is well perceived in literature that α-dicarbonyl compounds are the central intermediates in the non-enzymatic browning reaction, and that most advanced products can mechanistically be explained by their participation. Today the term Maillard reaction includes amino compound interactions with α-hydroxycarbonyls and α-dicarbonyls regardless of their origin. Major alternative sources include fat oxidation and fermentative processes (*2*, *3*).

Despite their importance published research on Maillard-dicarbonyls is rather confusing. Trapping reactions are needed for quantification, but derivatization was done both continuously and after end of incubation, and with different reagents. In addition a vast number of different, not compatible model reaction systems was applied monitoring different structures. There are, to the best of our knowledge, only very few publications to experimentally proof selected mechanistic relationships, most schemes still remain largely hypothetical. Therefore to gain further insights on dicarbonyl formation, we focused on a simple aqueous setup at 50°C and monitored all major structures with *o*-phenylenediamine as the trapping reagent.

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Experimental

The following reference dicarbonyls were prepared as reported recently in literature: 1-deoxyglucosone, glucosone, 3-deoxyglucosone (*4*). Maillard reaction mixtures were as follows. A solution of dicarbonyls or D-glucose (unlabeled and 1-/6-^{13}C-labeled) or D-maltose (42 mM) and L-lysine (42 mM) in phosphate buffer (0.1 M, pH 7.4) was heated at 50°C for 7 days. Deaeration was achieved by gassing with argon and the addition of diethylenetriaminepentaacetic acid (1 mM). Reactions were performed in presence of OPD (5 mM) for isolation of reference quinoxalines (continuous trapping method). For the post-incubation trapping method, OPD was added at the time of sampling (5 mM for glucose/maltose mixtures and 42 mM for dicarbonyl mixtures).

For isolation of reference quinoxalines reaction mixtures were first extracted with ethyl acetate. Both the water and the organic layer were concentrated and subjected to multilayer countercurrent chromatography with water as the mobile and ethyl acetate as the stationary phase ((*5*), Figure 1). From the individual peaks (A-H), pure material could be obtained either directly or after preparative HPLC on RP-18.

Quantification from post-incubation trapping was done on RP18-HPLC with UV-detection (Figure 1). Incorporation from ^{13}C-labeled sugars was verified by LC/MS/MS (Applied Biosystem 4000 Q Trap) in the enhanced resolution mode.

Results and Discussion

Trapping α-Dicarbonyls with *o*-Phenylenediamine

α-Dicarbonyl compounds are too reactive to allow direct detection. Therefore a variety of different trapping reagents has been employed to generate stable analytes, such as hydroxylamines, hydrazines, cysteamine, aminoguanidine and *o*-diaminobenzene derivatives (*4*). All reactions have in common that amines are used to trap the carbonyl group of dicarbonyls by condensation. The most frequently used trapping reagent is *o*-phenylenediamine (OPD), which gives stable quinoxalines (Figure 2). It becomes clear that although the trapping is needed for isolation and quantification there are two major disadvantages. First, significant amounts of amines are added to the particular research system, which will alter Maillard-reaction pathways by (i) trapping important intermediates, by (ii) acting as a Maillard-reactant itself, and by (iii) enhancing acid/base catalyses. Second, especially the *o*-diaminobenzene derivatives are highly redox active structures and therefore impose major oxidative stress. As a result for the degradation of the Amadori product at physiological conditions, OPD led to false glucosone formation. Two different modes of trapping have been used, continuous trapping and post-incubation trapping. In the first setup the trapping reagent is added immediately to Maillard-reactions and is therefore highly prone to above artifact formation. Still, it was useful in the present study to generate sufficient amounts of quinoxalines for isolation of authentic references and also to roughly estimate the total amount formed of a particular structure. In the post-incubation trapping OPD was added at various time points and re-incubated under carefully controlled conditions for a short time. This setup was used for mechanistic inquiries, as it minimizes adulterations.

Figure 3 clearly depicts the differences. With continuous trapping all structures accumulated with time, and glucosone was the far most abundant dicarbonyl under aeration with 4.5 mol% glucose after 7 days. Under de-aeration this yield decreased to 0.6 mol% and 1-deoxyglucosone increased from 0.7 to 2.4 mol%. The yields of 3-deoxyglucosone didn´t respond to the presence of oxygen (both 0.5 mol%). Post-incubation trapping revealed a totally different picture. While stable dicarbonyls as 3-deoxyglucosone still increased over time, glucosone and 1-deoxyglucosone levels increased to a maximum and then decreased again, however at significantly lower concentrations compared to continuous trapping. This means that highly reactive reductone structures can now degrade to follow-up products and extinct, once their precursors exhaust. The difference for the 1-deoxy- to the 3-deoxyisomeres in both trapping methods was reproduced for all derivatives measured.

Mechanisms Leading to Sugar Fragmentation

Several dicarbonyls with a C2-C5 carbon backbone were detected in glucose and maltose reactions, which must originate from scission of the original sugar skeleton. There are four major mechanisms proposed for sugar fragmentation in literature, retro-aldol cleavage, hydrolytic α-dicarbonyl cleavage, oxidative α-dicarbonyl cleavage and hydrolytic β-dicarbonyl cleavage (Figure 4). Aldol-condensation and retro-aldol-fragmentation reactions have been reported very early in Maillard chemistry, e.g. to explain the formation of acetic acid via glycolaldehyde from fragmentation of 1-deoxyglucosone (*6*). The coinciding generation of aldehydes and carboxylic acids of compatible carbon length lead to another fragmentation hypothesis by intramolecular disproportionation, i.e. hydrolytic α-dicarbonyl cleavage (*7*, *8*). However, an in-depth study revealed for the degradation of 2,3-pentanedione no aldehydes matching the formation of acetic acid and propanoic acid and ruled out this mechanism for aqueous Maillard systems (*9*, *10*). On the other hand by use of ^{17}O-NMR and $^{18}O_2$ enriched reaction mixtures, oxidative α-dicarbonyl cleavage leading to equimolar yields of corresponding carboxylic acids was proved as a minor pathway to proceed via

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a Baeyer-Villiger type rearrangement. As the major fragmentation mechanism hydrolytic β-dicarbonyl cleavage was established from 2,4-pentanedione and 1-deoxyglucosone reaction mixtures.

The later finding was extended by our investigations for an aqueous set up at 50°C. Reaction mixtures of authentic 1-deoxyglucosone and α-alanine gave up to 65 mol% of acetic acid independent from the presence of oxygen and up to 16 mol% of erythrulose (*11*). This and other C4-sugars (1-deoxythreosone, 3-deoxythreosone, threosone) stem from isomerization of the C4-1,2-enediol resulting from the hydrolyic β-cleavage (Figure 5, 2.). In further support for this single main fragmentation mechanism, acetol and glyceric acid were quantified in substantial amounts. However, we were not able to identify erythronic acid as the probe for oxidative α-dicarbonyl cleavage (Figure 5, 1.). In addition retro-aldol cleavage could be ruled out as a major mechanism for two reasons (Figure 5, 3.). First, only negligible amounts of methylglyoxal could be detected. Second, although 1-deoxythreosone and glycolaldehyde were quantified in significant amounts from 1-deoxyglucosone, incubations with ^{13}C-labeled glucose revealed that all C4-fragments originate strictly from the C3-C6-region of the original carbon backbone. Thus, retro-aldol reactions may not be of relevance at neutral pH and 50°C.

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Maillard-Glucose Intermediates

Table I lists the concentrations of the major α-dicarbonyls measured as quinoxalines by the post-incubation trapping method. Dicarbonyls with the intact original carbon skeleton of glucose reacted different to the presence of oxygen in the reaction mixtures. 3-Deoxyglucosone was formed completely independent and, more importantly, accumulated during time. This underlines the relative stability of this compound compared to glucosone and to 1-deoxyglucosone, which was further supported by differences in half-life in incubations of independent synthesized material (40 h vs. 8 h vs. 0.5 h). Obviously, reductones due to the given mobility of the carbonyl functions by isomerization are quickly degraded to other products. Here oxidation was of great impact on the amounts detectable. Glucosone requires oxidation for formation along the glucose – Schiff base – Amadori product road. 1-Deoxyglucosone is formed non-oxidatively via 2,3-enolization and subsequent water elimination, but as a reductone will be immediately oxidized to degradation products. Lederer´s glucosone (*N*^{6}-(3,6-dideoxyhexos-2-ulos-6-yl)-lysine) is a non reductone structure like 3-deoxyglucosone (*12*). Still, under aeration levels decreased by half. This must be explained by enolization along the entire carbon backbone, which is prerequisite to form the compound.

Glyoxal formation has been described by oxidative fragmentation mechanisms, focusing mainly on scission between C-2 and C-3 of the full carbon backbone (e.g. (*13–15*)). However, while the influence on oxidation was confirmed, our study with labeled glucose only attributed 49 % to those pathways. 51 % of glyoxal originated from the lower parts. Methylglyoxal was formed independent from aeration in our reaction mixtures. This is in agreement with literature which explains formation from C1-C3 mainly by retro-aldol reactions

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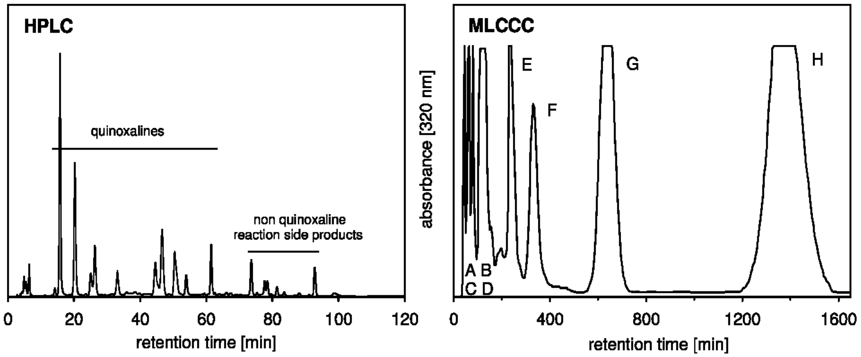

Figure 1. α-Dicarbonyls were quantified by HPLC and isolated by MLCCC

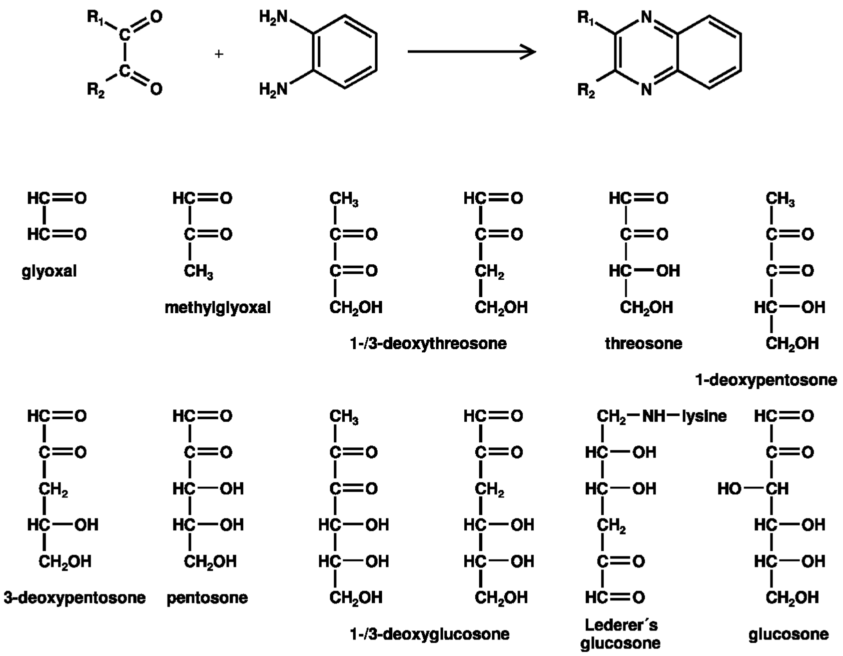
R₁
R₂
H₂N
H₂N
N
N
glyoxal
methylglyoxal
1-/3-deoxythreosone
threosone
1-deoxypentosone
3-deoxypentosone
pentosone
1-/3-deoxyglucosone
CH₂–NH–lysine
Lederer's glucosone
glucosone

Figure 2. OPD reacts with α-dicarbonyls to form quinoxalines (structures shown were isolated and quantified from glucose reaction mixtures)

from 1- and 3-deoxyglucosone (e.g. (*16*, *17*)). On the other hand, this is in contrast to label incorporation, which assigned only 32 % to the upper part. In addition, reactions of authentic both 1- and 3-deoxyglucosone yielded only negligible amounts of methylglyoxal. It therefore must be assumed that major quantities arise from the C4- and C5-sugar fragments described below.

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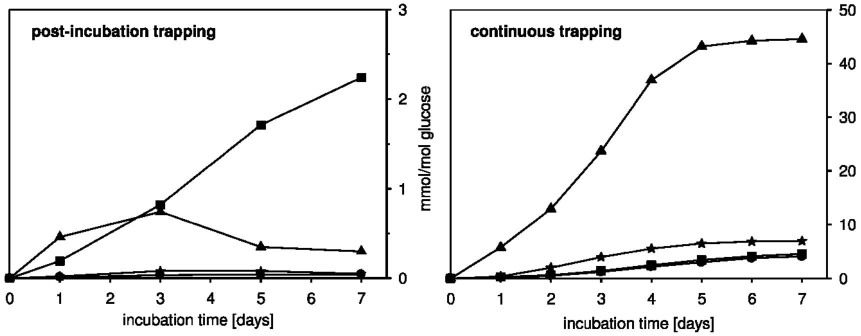

*Figure 3. Comparison of post-incubation trapping with continuous trapping in glucose incubations (aerated): quinoxalines of ▲ glucosone, * 1-deoxyglucosone, ■ 3-deoxyglucosone, ● Lederer's glucosone*

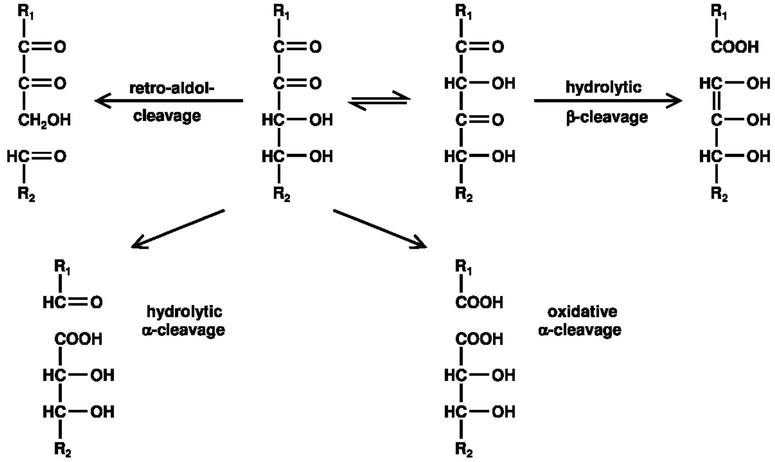

Figure 4. Mechanisms of sugar fragmentation

C4-Dicarbonyls (1-/3-deoxythreosone, threosone) and C5-dicarbonyls (1-/3-deoxypentosone, pentosone) strictly originated from the lower parts of the glucose skeleton. Threosone derivatives could unequivocally be assigned to 1-deoxyglucosone, underlining the importance of the hydrolytic β-cleavage (*11*). Interestingly, 1-and 3-deoxypentosone showed a similar relationship as their C4-analogues, but in contrast were elevated under aeration. This called for a precursor, which relays on oxidative pathways. Indeed, when glucosone was incubated, C5-dicarbonyls were formed in significant amounts in the same ratio as in glucose reactions. We therefore propose the same mechanism of hydrolytic β-cleavage (Figure 6). Glucosone isomerizes to the 1,3-tautomer which, after hydration, cleaves off formic acid to give an C5-1,2-enediol. Water elimination yields 3-deoxypentosone, and after 2,3-enolization 1-deoxypentosone. In further support we were able to detect traces of pentosone, which is the direct oxidation product of that enediol.

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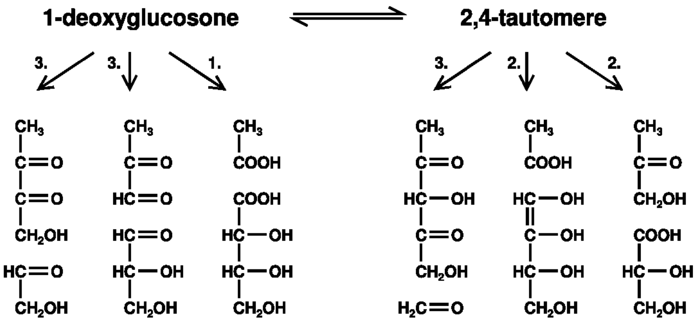

Figure 5. Fragmentation products from 1-deoxyglucosone based on 1. oxidative α-cleavage, 2. hydrolytic β-cleavage and 3. retro-aldol cleavage

Table I. Formation of Maillard-glucose-α-dicarbonyls quantified as quinoxalines by post-incubation trapping after 7 days

α-dicarbonyl	*aerated*	*de-aerated*
	mmol/mol glucose	
1-deoxyglucosone	0.05	0.22
3-deoxyglucosone	2.24	2.19
Lederer´s glucosone	0.04	0.23
glucosone	0.30	0.16
1-deoxypentosone	0.03	0.01
3-deoxypentosone	0.21	0.10
pentosone	traces	not detected
1-deoxythreosone	0.05	0.11
3-deoxythreosone	0.15	0.23
threosone	0.25	0.08
methylglyoxal	0.08	0.08
glyoxal	0.12	0.06

Maillard-Maltose Intermediates

A total of 19 dicarbonyl quinoxalines was isolated from maltose reaction mixtures covering a range of the complete carbon backbone to C3- and C2-fragments. The following structures were, in the main, related to the glucose chemistry described above: glyoxal, methylglyoxal, 1-/3-deoxythreosone, 1-/3-deoxyglucosone and glucosone. Besides the formation of maltosone and 1-/3-deoxymaltosone other fragments were typical for disaccharide degradation: glyoxylic acid, diacetyl, 3-deoxypentosone, 3,4-dideoxypentosone, 5,6-dihydroxy-2,3-dioxohexanal (1,2,3-tricarbonyl), 1,4-dideoxyglucosone, *N*^{6}-(5,6-dihydroxy-2,3-dioxohexyl)-lysine (1-amino-1,4-dideoxyglucosone) and 5,6-dihydroxy-2,3,4-trioxohexane (2,3,4-tricarbonyl). From maltose the

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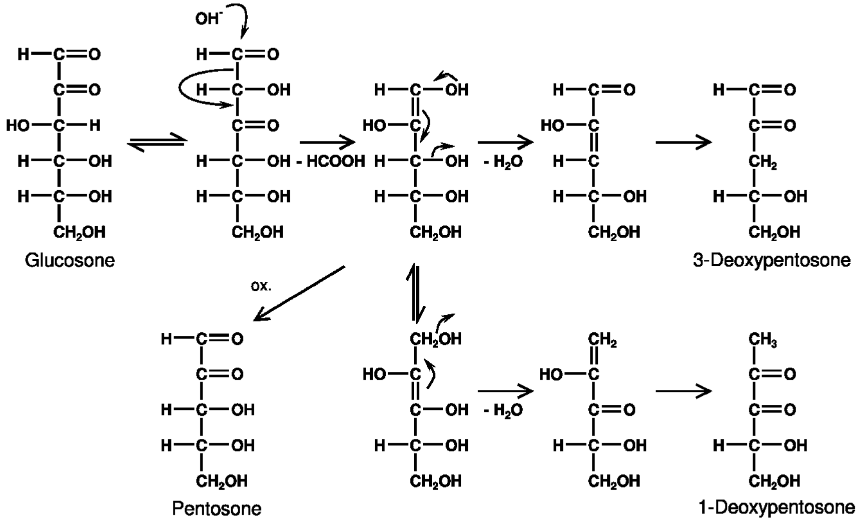

Figure 6. Formation of C5-fragments from glucosone via β-dicarbonyl cleavage

formation of pentosone fragments was completely different to that from glucose. While 1-deoxypentosone could not be detected, 3-deoxypentosone was elevated under aeration. Starting from maltosone as the most likely precursor, the lack of 1-deoxypentosone can be explained according to Figure 6. After hydrolytic β-cleavage the 1,2-pentenediol will immediately cleave off glucose from the former position 4. The good leaving tendency of glucose will thus not allow further isomerization to the 2,3-enediol.

3,4-Dideoxypentosone is a product of reduction and has been linked to the formation of 4-deoxyglucosone (*18*). However in our study, the concentration of 4-deoxyglucosone was 10-fold less than that of 3,4-dideoxypentosone (0.4 mmol/ mol maltose) and did not respond to aeration. Within the C6-dicarbonyl fragments there were further structures calling for redox processes (Table II).

Paradoxically, 1,2,3-tricarbonyl was formed preferentially under de-aeration paralleled by 1-amino-1,4-dideoxyglucosone and 1,4-dideoxyglucosone. By definition, 1,2,3-tricarbonyl must originate from oxidation in contrast to 1,4-dideoxyglucosone from reduction. Under aeration quantities for 1-amino-1,4-dideoxyglucosone and 1,4-dideoxyglucosone were negligible. The only reaction that allows both reduction and oxidation in absence of oxygen is disproportionation. We therefore suggest the mechanism depicted in Figure 7 to explain the coinciding structures. Maltose first forms the Amadori product under incorporation of the amine. The 1,2-enediol will eliminate water to yield 3-deoxymaltosone. The 2,3-enediol has two choices. Loss of the amine will give 1-deoxymaltosone, but the major route will follow elimination of glucose because of its better leaving properties to result in 1-amino-1,4-dideoxyglucosone. This nitrogen-analogue reductone reacts intermolecular to 1,2,3-tricarbonyl by oxidation paralleled by an amino-hexulose via reduction. This intermediate then yields 1,4-dideoxyglucosone after elimination of the amine.

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Table II. Formation of selected C6-Maillard-maltose-α-dicarbonyls quantified as quinoxalines by post-incubation trapping after 7 days

α-dicarbonyl	*aerated*	*de-aerated*
	mmol/mol maltose	
4-deoxyglucosone	0.05	0.04
1,2,3-tricarbonyl	0.51	2.57
1-amino-1,4-dideoxyglucosone	0.08	0.43
1,4-dideoxyglucosone	not detected	1.65

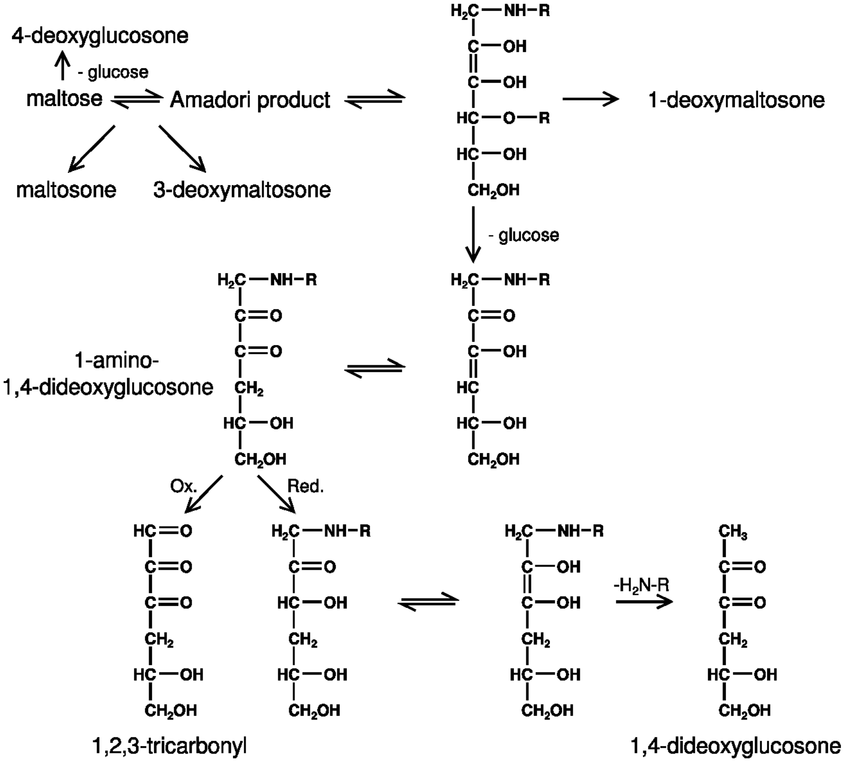
4-deoxyglucosone
- glucose
maltose
Amadori product
maltosone
3-deoxymaltosone
1-deoxymaltosone
- glucose
1-amino-
1,4-dideoxyglucosone
Ox.
Red.
-H₂N-R
1,2,3-tricarbonyl
1,4-dideoxyglucosone

Figure 7. 1-Amino-1,4-dideoxyglucosone plays a major role in maltose redox reactions

Conclusions

The current study represents a major step forward to understand the formation of reactive Maillard-intermediates from glucose and maltose. In glucose reactions glucosone and 1-deoxyglucosone were verified as the major source of shorter-chained products via hydrolytic β-dicarbonyl cleavage. Maltose reactions verified intermolecular redox reactions as a second important mechanism of reductone structures with 1-amino-1,4-dideoxyglucosone as the important player. Further studies are needed to fully understand redox Maillard-chemistry.

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Chapter 5

Generation of 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone during Extrusion Cooking: A Multidisciplinary Approach

**Tomas Davidek,[1] Silke Illmann,[2] Andreas Rytz,[3] Hélène Chanvrier,[1]
Greet Vandeputte,[1] Heike P. Schuchmann,[2] Imre Blank,[1]
and Josef Kerler*,[1]**

[1]Nestle Product Technology Centre Orbe, Nestec, Ltd., 1350 Orbe, Switzerland
[2]Food Process Engineering, Karlsruhe University, 76131 Karlsruhe, Germany
[3]Nestle Research Centre, 1000 Lausanne 26, Switzerland
***Josef.Kerler@rdor.nestle.com**

The generation of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) has been studied during extrusion cooking using a holistic approach: modulating caramel flavor of the extruded product while mastering other product characteristics such as texture, color, and formation of undesirable components. Using a fractional factorial experimental design, all main effects and two factor interactions of three recipe and five process parameters were investigated. HDMF generation was affected by both recipe parameters (presence of lysine, phosphate) and extrusion parameters (temperature, moisture). In contrast, the change in recipe parameters permitted HDMF modulation without any impact on texture. In addition, lysine led to an increase in HDMF content. Other parameters like pH did not affect HDMF generation, but were found to be a means to attenuate color development. Such a comprehensive and multidisciplinary approach has been found very powerful when it comes to flavour modulation in complex food systems.

In Controlling Maillard Pathways To Generate Flavors; Mottram, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

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Introduction

Extrusion cooking is gaining increasing importance in food industry as a cost efficient way of manufacturing numerous food items including cereal products. It permits in one processing step to perform several unit operations such as mixing, transporting, cooking and texturing. However, desirable flavor characteristics associated with conventionally cooked cereals do not develop to the same extent during extrusion cooking. Several academic studies have recently been published concerning the effect of extrusion parameters on the volatile formation (*1–3*). Extrusion temperature, moisture, pH and composition of the feedstock were shown to have the highest effect on the volatiles formed. Unfortunately, no or very limited information is available on the formation of odor-active compounds or the organoleptic qualities of the extrudates.

The use of specific precursors and ingredients is of particular interest to achieve flavor modulation. However, knowledge has to be built-up concerning the effect of precursors and extrusion parameters on flavor development and on other key product attributes such as color and texture. This study is based on an experimental design targeting the formation of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) from rhamnose (a well-known precursor of HDMF) (*4–6*) to impart caramel-type flavor notes upon extrusion. It will be shown how HDMF generation during extrusion can be optimized as a function of both recipe and extrusion parameters, while considering other product characteristics such as for example texture (e.g. viscosity), color and the formation of other compounds.

Experimental

Materials

L-Rhamnose monohydrate (>99%) (Kaden Bio-chemicals, Hamburg, Germany); L-lysine·HCl (>99%) (Aminolabs, Hasselt, Belgium); monosodium phosphate anhydrous (>98%), disodium phosphate anhydrous (>98%) (Thermophos International BV, Vlisingen, The Netherlands), rice flour (Remy Industries N.V., Leuven-Wijgmaal, Belgium); calcium carbonate (Brenntag Schweizerhall, Basel, Switzerland); 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF, 99.6%) (Givaudan, Dübendorf, Switzerland); $^{13}C_2$-HDMF (isotopic purity >99%) (Aroma Lab, Planegg, Germany); methanol (>99.9%) (Merck, Darmstadt, Germany); methyl acetate (99.5%), sodium sulfate anhydrous (>99%) (Fluka, Buchs, Switzerland).

Extrusion

The trials were performed on a BC 21 extruder (Clextral, Firminy, France) using a rice flour based model recipe. As recipe and process parameters strongly interact and the “Maillard reaction” comprises several sequentially or parallel interacting reactions of unknown kinetics (*7*), selected parameters of theoretical relevance were varied according to an experimental design by applying a fractional factorial design leading to 32 trials. These were recipe parameters (rhamnose/lysine ratio: 1:0, 3:1; phosphate concentration: 0.035 mol/kg, 0.134 mol/kg; moisture: 17%, 20%, 23%; pH 6.4, 7.7) and extrusion parameters (screw length: 500 mm, 700 mm; screw speed: 300 rpm, 400 rpm 500 rpm; product die temperature: 120°C, 135°C, 150°C; method of precursor addition: slurry, dry). Rhamnose and lysine were either added as such into a dry mix (dry addition) or were dissolved in water and injected into extruder (slurry preparation). The extruded products were dried in Minimat ZIBO M oven (Wiesheu, Germany) and milled in a MGI-628 mill (Frewitt, Switzerland).

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Rhamnose Analysis

Ground product (2 g) was suspended in hot water (75 mL, 70°C) and extracted (30 min) in a volumetric flask (100 mL). After the extraction, the volume of the extract was adjusted to 100 mL with water, filtered through a double layer filter (Acrodisc PF Syringe Filters, 0.8/0.2 µm/25 mm) and analyzed by high performance anion exchange chromatography (HPAEC). Quantitative data were obtained using a calibration curve.

HPAEC analyses were performed on a Dionex ion chromatography system (DX500, Dionex, Sunnyvale, USA) as described in (*8*) using following modification: The separation was accomplished on a 250 mm x 4 mm i.d. CarboPac PA100 anion exchange column (Dionex) and a 50 mm x 4 mm i.d. CarboPac PA100 guard column (Dionex). Isocratic separation used water (A) and NaOH (100 mmol/L, B): 88 % A and 12 % B as a mobile phase at a flow rate of 1 mL/min for 18 min.

Analysis of HDMF

Ground product (1 g) was suspended in cold water (50 mL), spiked with the internal standard ($^{13}C_2$-HDMF, 150 µL, 3 µg/ml methanol) and extracted (1 h) using a magnetic bar stirrer. After extraction, the extract was filtered through a PURADISCTM 25 PP (polypropylene, 0.45 µm/25 mm) and a double layer filter (Acrodisc PF Syringe Filters, 0.8/0.2 µm/25 mm). An aliquot of filtrate (2 mL) was cleaned-up by solid phase extraction (SPE) as described (*9*), however HDMF and internal standard were eluted with methyl acetate (2 mL).

GC-MS analyses were performed on a Finnigan Trace gas chromatograph coupled to a Finnigan Trace mass spectrometer (ThermoQuest, Italy) equipped with MPS2 autosampler (Gerstel, Switzerland). The separation was achieved on a ZebronWAX capillary column (30 m x 0.25 mm, film thickness 0.25 µm, Phenomenex, USA) using helium as a carrier gas with a constant flow of 2 mL/min. Samples were introduced via splitless injection at 240°C (1 µL). The oven temperature program was 40°C (2 min), 40°C/min to 120°C, 6°C/min, to 185°C, 10°C/min to 240°C (10 min). The temperature of the ion source was 200°C. Mass spectra in the EI mode were generated at 70 eV over a mass of 30 to 220 Da. Quantification of HDMF was performed in the scan mode by measuring the molecular ions of HDMF (*m/z* 128) and that of the internal standard $^{13}C_2$-HDMF (*m/z* 130).

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Color Analysis

This was performed on a ColorFlex spectrophotometer (Hunterlab, USA) according to the standardized principle of CIELAB L*a*b* system (*10*).

Viscosity Measurement

The powder (21 g) was reconstituted in 170 ml preheated milk (~70°C) and the viscosity was measured using a Rapid visco analyser RVA-4 (Newport scientific). The measurement was carried out for 10 minutes at 50°C and with a constant shear rate of 50 min^{-1}. From the curve, the maximum of viscosity reached within the 10 min (visco max) was extracted.

Results and Discussion

Data analysis of all trials revealed that the level of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) formation was independent of the specific mechanical energy (SME; mainly depends on factors such as screw speed and torque) of the extruder. On the other hand, all other product attributes could be correlated either with the HDMF level or with SME. The correlation matrix is shown in Figure 1.

HDMF was positively correlated with color development (increase of *a* and *b*, and decrease of *L*) and negatively correlated with the level of non-reacted rhamnose. This was due to the fact that HDMF was generated from rhamnose via Maillard-type reactions and that these were accompanied by color development. This also means that a deeper understanding is needed of the effects of processing parameters on the flavor and color development to decouple flavor generation from color formation. As expected, HDMF formation in these concentrations was not correlated with the viscosity of the reconstituted product. The latter attribute was highly correlated with the SME value, as this in turn is responsible for amylopectin degradation (*11*). This means that it should be possible to enhance generation of HDMF without changing the viscosity.

Figure 2 shows estimates of the effect of each recipe/processing parameter on the HDMF level. The higher the absolute value of the main effect the bigger the impact of a given parameter on the product attribute.

The HDMF yield was influenced both by recipe parameters and extrusion parameters. It was increased most markedly by the addition of lysine. For example, the yield of HDMF after extrusion was by 7 mol% higher in the presence of lysine as compared to its absence. This indicates that in extruded products the HDMF formation were favored in the presence of amino acids. Interestingly, when also examining acrylamide levels in these extruded products, it has been shown that its formation could be suppressed in the presence of lysine as well as at lower pH (data not shown). As a result, HDMF yields can be optimized while mitigating acrylamide formation at the same time.

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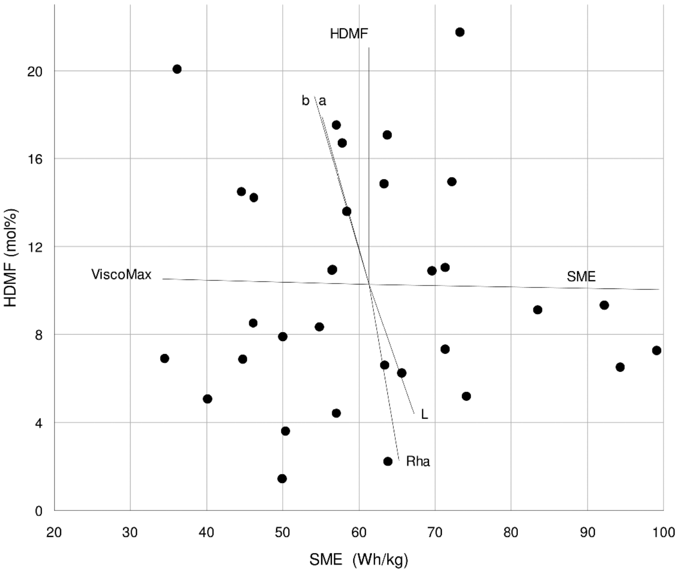

Figure 1. HDMF yield of all products (bullets) plotted over the SME including the visualization of the correlation matrix (lines).

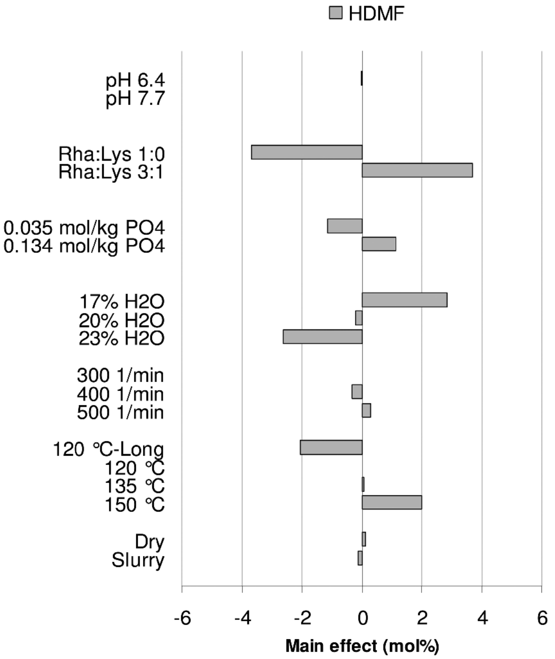

Figure 2. Main effects of recipe/process parameters on the HDMF yield. The main effect is expressed as the difference of the overall mean of HDMF level of all (32) trials and the mean HDMF level of all trials performed at a given parameter (e.g. at 17% moisture).

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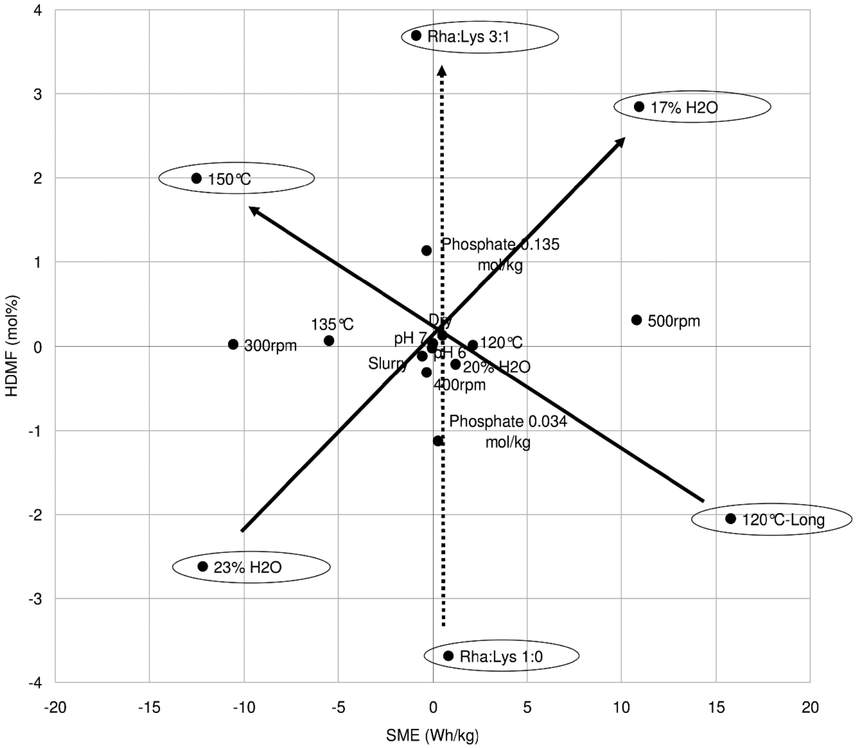

Figure 3. Main effects of HDMF plotted versus main effects of SME.

Moisture, temperature and phosphate level were other parameters influencing the yield of HDMF. Low moisture, high extrusion temperature and high phosphate level favored the formation of HDMF. This confirms the results reported in the literature that phosphate ions favor HDMF formation (*6*, *12*), as well as that high temperature and low moisture conditions, enhance flavor generation during extrusion cooking (*2*, *13*, *14*).

Screw speed, pH and the method of precursor addition (dry/slurry) had negligible effect on HDMF formation. These results confirm those of Parker et al. (*15*) who observed only marginal effects of screw speed on the flavor formation. On the other hand, the results of Riha and Ho (*13*) were not confirmed, i.e. higher concentration of all volatile compounds by slurry addition compared with dry addition.

Plotting the main effect of HDMF versus main effects of the SME permits visualizing how the different recipe/process parameters affect both values (Figure 3). Modulating the level of HDMF could be accomplished by either varying recipe parameters (lysine, phosphate) or process parameters (temperature, moisture). However, temperature and moisture (diagonal arrows) affected both HDMF levels and SME (e.g. both levels increase with increased moisture), indicating that changes of several product attributes occurred. As the viscosity was negatively correlated with SME, a decrease of viscosity was observed. The level of lysine or phosphate did not impact SME, thus these parameters can be used to modulate the flavor of the final product without affecting too much the other product attributes such as viscosity (vertical arrow).

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Chapter 6

Hydroxycinnamic Acid – Maillard Reactions in Simple Aqueous Model Systems

Marlene R. Moskowitz[a] and Devin G. Peterson[*,b]

[a]Department of Food Science, The Pennsylvania State University, University Park, PA 16802
[b]Department of Food Science and Nutrition, University of Minnesota, 147 FScN, St. Paul, MN 55108
[*]dgp10@psu.edu

The hydroxycinnamic acids (HCAs) in foods have been suggested to contribute to the flavor properties by multiple mechanisms. In this paper we investigate the reactivity of hydroxycinnamic acids in simple aqueous Maillard model systems on flavor development. Using isotope labeling techniques, the hydroxycinnamic acids were reported to form adducts with transient Maillard reaction flavor precursors, hexose fragments, as well as with amino acids and/or amino acid reaction products. The generation of Maillard-type volatile compounds, such as pyrazines, cyclotene, diacetyl, 2-acetyl-1-pyrroline in the model systems, was also suppressed by addition of HCAs. In summary, the HCAs were reported to alter the mechanisms of Maillard flavor development in these aqueous model systems.

Introduction

The Maillard reaction is a well known mechanism of flavor development in food products. The generation of Maillard-type flavor compounds is desirable for many foods such as bread, chocolate and coffee (*1*), but can also produce non-pleasant attributes in other products, such as in ultra high temperature (UHT) milk (*2*). The basic reactants for the Maillard reaction include a carbonyl and an amine typically provided by reducing sugars and amino acids or peptides in foods (*1*). The types of carbonyls and amines in foods as well as the reaction conditions, such

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as water content/activity, temperature, and pH are widely reported to influence the products formed via this reaction (*1*). In addition to the precursors which define the reaction (carbonyl and amine), food molecules such as phenolic compounds and lipid oxidation products have also been reported to influence the mechanisms of the Maillard reaction (*3*–*5*).

The hydroxycinnamic acids (HCAs) are ubiquitous in plant material and are the predominant phenolic compounds in products such as grain and coffee beans. The HCAs have been reported to alter the flavor properties of related foods by multiple mechanisms, including Maillard-type flavor development (*6*). The objective of this paper was to investigate the reactivity of hydroxycinnamic acids on flavor development in simple aqueous Maillard model reactions. We have previously reported on the reactivity of hydroxycinnamic acids in low moisture glucose-glycine models (*7*). Based on preliminary experiments in aqueous models, the reactivity of the hydroxycinnamic acids was found to be different in comparison to low moisture model systems and therefore was further investigated.

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Materials and Methods

Chemicals

Glucose, glycine, proline, ferulic acid, cinnamic acid, caffeic acid, p-coumaric acid, sodium phosphate monobasic, sodium phosphate dibasic, hydrochloric acid, sodium hydroxide, *n*-dodecane, pyrazine, methylpyrazine, 2,5-dimethylpyrazine, 2,3-butanedione, 3-hydroxy-2-butanone, furfuryl alcohol, cyclotene, glyoxal, glycolaldehyde, methylglyoxal and hydroxyacetone were purchased from Sigma Aldrich (St. Louis, MO). Anhydrous sodium sulfate, diethyl ether, methanol, ammonium acetate were purchased from EMD Chemicals (Gibbstown, NJ). $^{13}C_6$-glucose, $^{13}C_2{}^{15}N$-glycine was purchased from Cambridge Isotope Laboratories (Andover, MA).

Model Reaction Systems

Equimolar concentrations (10 mM) of reactants (see Table I) were reacted in 100 mL of a 0.1 M, pH 7.0 phosphate buffer solution at 125°C for 30 min in a 600 mL Parr Reactor (model 4563, Parr Instrument Co., Moline, IL) under constant stirring and immediately cooled to approximately 30°C and prepared for further analysis.

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Table I. Model System Compositions

Reactants [1]	*Reactant Concentration (mM)*					
	Model A	*Model B*	*Model C*	*Model D*	*Model E*	*Model F*
glucose	10	10	5	10		
amino acid [2]	10	10	10	5	10	10
phenolic acid [3]		10	10	10		10
$[^{13}C_6]$glucose			5			
$[^{13}C_2, {}^{15}N]$glycine				5		
sugar fragment [4]					10	10

[1] Reactants were dissolved in 0.1 M phosphate buffer, reacted at 125°C for 30 min (does not include reactor heating time to 125°C) and cooled down to 30°C prior to sample preparation. [2] amino acid; glycine or proline. [3] phenolic acid; ferulic, cinnamic, caffeic, or p-coumaric acid. [4] sugar fragment; glyoxal, glycolaldehyde, methylglyoxal, or hydroxyacetone.

Gas Chromatography/Mass Spectrometry-Electron Impact Analysis (GC/MS-EI)

Ninety mL of A, B, E and F model reaction mixtures were extracted three times with 25 mL of diethyl ether containing 0.8 ppm *n*-dodecane as the internal standard. The organic extract was dried with anhydrous sodium sulfate, filtered and concentrated down to 0.5 mL using a spinning band distillation apparatus (model 800, B/R Instruments, Easton, MD). Distillates were subsequently analyzed with a 6890 Agilent GC equipped with a flame ionization detector and 5973 Mass Selective Detector (Agilent Technologies, Inc.; Santa Clara, CA), a DB-Wax capillary column (30 m x 0.25 mm ID with 0.25 µm film thickness, Agilent Technologies, Inc.; Santa Clara, CA). One µL of sample was injected in split mode, with inlet temperature set to 200°C and detector temperature of 250°C. MS analysis was done with Electron Impact Ionization, scanning between 35 and 250 *m/z*. The oven/time program was as follows: 30°C for 2 minutes, ramped by 5°C/min to 180 °C, ramped by 35°C/min to 230°C, and then held for 6 min.

Liquid Chromatography/Mass Spectrometry-Electrospray Ionization Analysis (LC/MS-ESI)

Nine mL of A-F model reaction mixtures were fractioned on 0.5 g C_{18} Sep-pak cartridge (Supelclean™ LC-18; Supelco, Bellefonte, PA), preconditioned with 5 mL each of methanol, then reverse osmosis water. Ten µL of an internal standard (300 ppm butylparaben in methanol) was added to the sample prior to SPE clean-up. The cartridge was washed with 5 mL of reverse osmosis water

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and then eluted with 2 mL of HPLC grade methanol under vacuum at a rate of 1 mL/min. The eluent was concentrated to approximately 1 mL under a stream of nitrogen and filtered through 0.45 μm PTFE tip filter. The concentrate was subsequently analyzed with a Shimadzu HPLC system (Shimadzu, Columbia, MD), consisting of two pumps (LC-10ADvp), an autosampler (SIL-10vp) and on-line degasser (DGU-14A). The system was interfaced to a Waters Micromass Quattro Micro mass spectrometer (Waters, Milford, MA). The samples were analyzed using an Ultra Aqueous C18 column (250 x 2.1 mm I.D.; 5 μm particle size; Restek; Bellefonte, PA) kept at 28°C using a column heater (model TCM; Waters, Milford, MA). The flow rate was 0.2 mL/min, with a linear concentration gradient of two mobile phase solvents A (10 mM ammonium acetate, pH 7.0) and B (methanol). The program began at 10% B in A (0 – 2 min) and then increasing B to 99% over 28 min (2 – 30 min) and held for 4 min (30 – 34 min) before decreasing to 10% (34 – 35) and held for 8 min (35 – 43 min). MS data was collected over the scan range of 120 to 500 *m/z* with a scan time of 1.0 s in either negative or positive ion mode.

Results and Discussion

The influence of four common food hydroxycinnamic acids (shown in Figure 1) on the generation of Maillard-type aroma compounds in a glucose/glycine model system is illustrated in Figure 2. In general the addition of hydroxycinnamic acids (HCAs) significantly reduced the generation of six of the seven main aroma compounds detected in these Maillard models. Differences in the level of suppression were also noted among the four structurally unique hydroxycinnamic acids analyzed; caffeic acid was the most reactive in suppressing aroma development.

To investigate the reactivity of HCAs on mechanisms of Maillard product generation in these models, two isotopic labeling techniques CAMOLA (CArbon MOdule LAbeling; model C) (*8*) and AAMOLA (Amino Acid MOdule LAbeling; model D) (*9*) were utilized as previously reported (*7*). Since ferulic acid is the most abundant phenolic acid found in wheat (*10*), it was selected for further analysis.

When ferulic acid was reacted with glucose and glycine (model B) an analyte with the predicted molecular weight of 210 Da (*m/z* 209 $[M-1]^-$) was detected (Figure 3ii); which was not reported in the control glucose/glycine reaction (Figure 3i, model A).

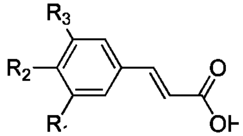

Figure 1. Chemical structures of select hydroxycinnamic acids, ferulic: R_1=OCH_3, R_2=OH, R_3=H; cinnamic: R_1=R_2=R_3=H; p-coumaric: R_1=R_3=H, R_2=OH; caffeic: R_1=R_2=OH, R_3=H

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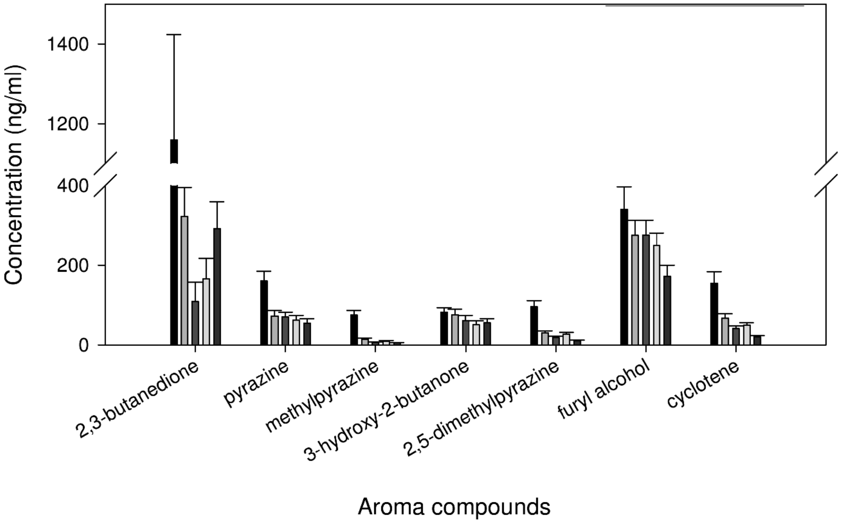

Figure 2. Concentrations of key volatile generated in aqueous model systems, 125 °C, pH 7; left to right Model A: glucose + glycine ■, Model B: glucose + glycine + phenolic acid (ferulic ■, cinnamic ■, p-coumaric ■, caffeic acid ■, respectively); 95% Confidence Interval shown

For the CAMOLA reaction (Figure 3iii), the plus 2 isotopomer (*m/z* 211) indicated this analyte consisted of an intact C2 glucose fragment, whereas for the AAMOLA technique no isotopomer was detected (Figure 3iv) or glycine was not part of this reaction product. Consequently this analyte consisted of a ferulic acid moiety and a two carbon glucose fragment. Two common two carbon sugar fragments of hexose sugars, glyoxal and glycolaldehyde, were reacted with ferulic acid and glycine (no glucose) and compared to the ferulic acid/glucose/glycine reaction for the generation of this phenolic-sugar fragment adduct (see Figure 4). Based on the chromatograms in Figure 4, glycolaldehyde generated a similar analyte 'fingerprint' as was generated in the glucose/glycine/ferulic acid model (Figure 4iii) and thus was identified as the sugar fragment moiety of this ferulic acid-Maillard reaction product.

Ferulic acid-Maillard reaction products consisting of both a glucose fragment and a glycine moiety were also detected in these models. Figure 5 illustrates an analyte detected in model B (Figure 5ii) but not in model A (Figure 5i) with the predicted molecular weight of 427 Da based on the *m/z* 428 $[M+1]^+$ ion. This analyte consisted of an intact three carbon glucose moiety (plus 3 isotopomer detected with *m/z* 431 ion, Figure 5iii) as well as an intact C and N skeleton of glycine (plus 3 isotopomer detected with *m/z* 431 ion, Figure 5iv). Similarly, to identify the source of the three-carbon fragment incorporated into the analyte observed in Figure 5ii, common three carbon hexose fragments, hydroxyacetone (Figure 6i) and methylglyoxal (Figure 6ii) were reacted with glycine and ferulic acid. Based on the single peak generated in the chromatogram (Figure 6ii and 6iii), methylglyoxal and glycine were identified as the reactants for this phenolic reaction product.

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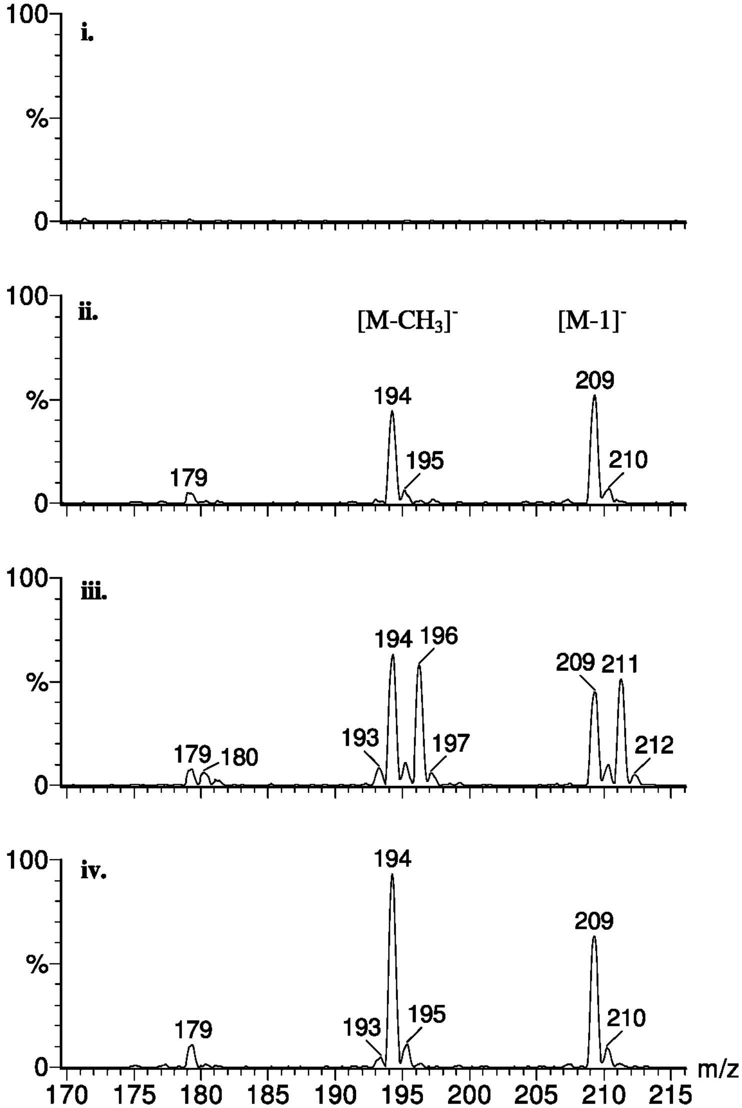

Figure 3. LC/MS-ESI (-ve) Spectrum of select analyte m/z 209[M-1]$^-$ generated in aqueous model systems, 125°C, pH 7: (i) Model A. glucose + glycine, (ii) Model B. glucose + glycine + ferulic acid, (iii) Model C. CAMOLA: $^{13}C_6$: $^{12}C_6$ glucose + glycine + ferulic acid, (iv) Model D. AAMOLA: glucose + $^{13}C_2$,^{15}N:$^{12}C_2$,,^{14}N glycine + ferulic acid; all at equivalent retention time

The observed reactivity of HCAs with sugar fragments or sugar fragment/amino acid products, which are known precursors of Maillard-type aroma compounds, provided a mechanism to define how HCAs altered Maillard chemistry and aroma development in these model systems. Considering HCAs are abundant in grains, additional model systems were analyzed for the influence of ferulic acid on the generation of 2-acetyl-1-pyrroline, an important aroma compound found in cereal products, such as bread (*11*). Methylglyoxal is a known precursor of 2-acetyl-1-pyrroline (*11*). These model systems (Models F-G, Table I) consisted of proline, as the amino acid source, and methylglyoxal with and without ferulic acid. The addition of ferulic acid to this model system resulted in a 39% decrease in the amount of 2-acetyl-1-pyrroline generated.

In summary, HCAs were reported to trap precursors of Maillard-type aroma compounds, such as two- and three-carbon sugar fragments and amino acid moieties, and consequently suppressed aroma development in these aqueous model systems. On-going research in our laboratory is focused on translating the findings of these model systems to flavor development in whole grain foods.

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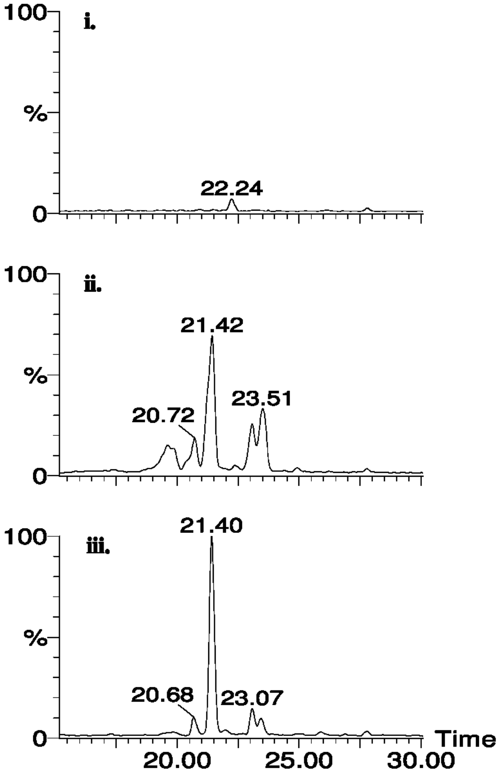

Figure 4. LC/MS-ESI(-ve) chromtogram of a 4-vinylguaiaol-glycolaldehyde adduct (m/z 209[M-1]⁻) generated in aqueous model systems, 125°C, pH 7: (i) Model F: glyoxal + glycine + ferulic acid; (ii) Model F: glycolaldehyde + glycine + ferulic acid (iii) Model B: glucose+glycine+ferulic acid

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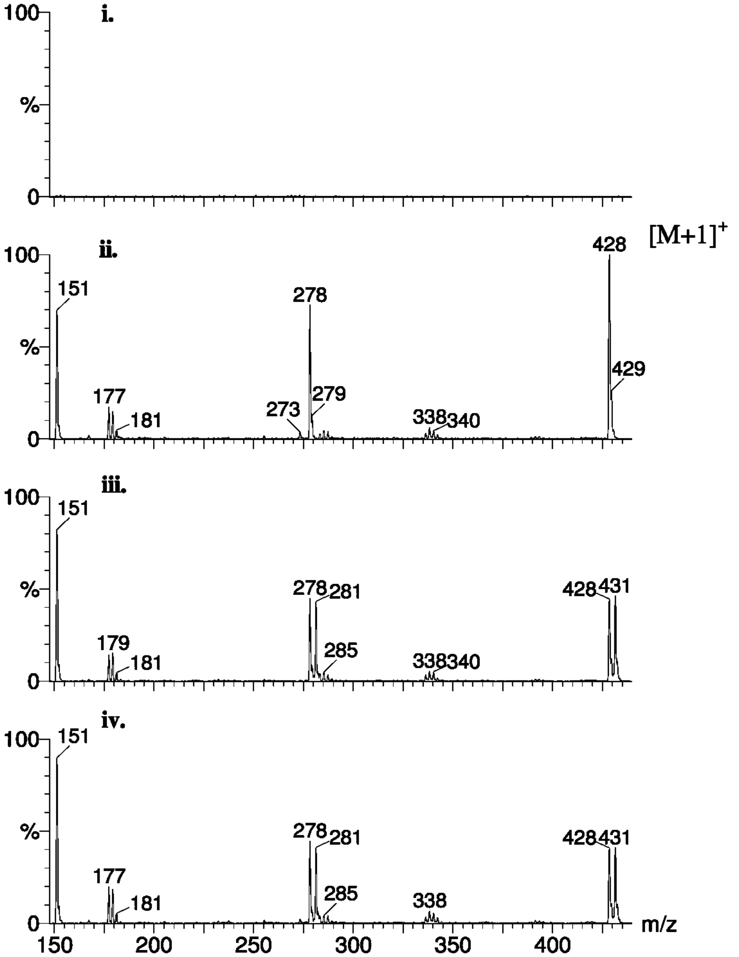

Figure 5. LC/MS-ESI (+ve) Spectrum of select analyte m/z 428[M+1]+ generated in aqueous model systems, 125°C, pH 7: (i) Model A. glucose + glycine, (ii) Model B. glucose + glycine + ferulic acid, (iii) Model C. CAMOLA: 13C6 : 12C6 glucose + glycine + ferulic acid, (iv) Model D. AAMOLA: glucose + 13C2,15N:12C2,14N glycine + ferulic acid; all at equivalent retention time.

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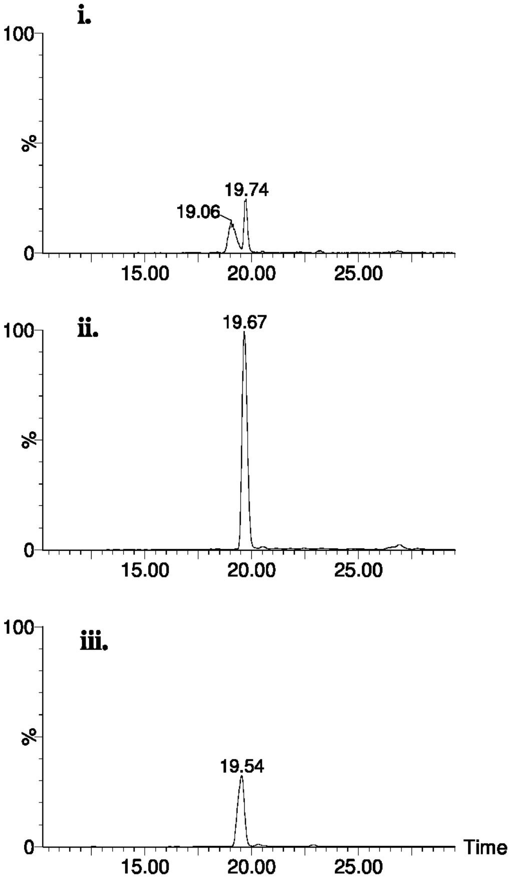

Figure 6. LC/MS-ESI(+ve) chromtogram of a 4-vinylguaiaol-methylglyoxal adduct (m/z 428[M+1]$^+$) generated in aqueous model systems, 125°C, pH 7: (i) Model F. hydroxyacetone + glycine + ferulic acid, (ii) Model F. methylglyoxal + glycine + ferulic acid, (iii) Model B. glucose + glycine + ferulic acid

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Chapter 7

Science and Serendipity: The Maillard Reaction and the Creative Flavorist

David A. Baines,*,[1] Sandra Bishara,[2] Jane K. Parker,[2] and Donald S. Mottram[2]

[1]Baines Food Consultancy, Ltd., Thornbury, Bristol BS35 2YN, United Kingdom
[2]Department of Food and Nutritional Sciences, University of Reading, Whiteknights, Reading RG6 6AP, United Kingdom
***db@bfc.demon.co.uk**

The chemical compounds formed in the Maillard reaction of relevance to meat flavor have been largely discovered over the past 25 years. Before that, flavor chemists would use a 'creative' approach to the formation of meat flavors, relying on a combination of serendipity, trial and error and cooking skills. Many of the early flavors produced by these methods were very good but now flavor chemists have a better understanding of the target chemicals needed to create particular flavors and can design reactions to generate and optimize these important compounds. Nevertheless the creative approach is still relevant today and combined with an understanding of the reaction mechanisms, can lead to the design of powerful and characteristic flavors.

A Historical Perspective

When Morton, Akroyd and May filed their landmark patent in 1960 it heralded the start of a new era in flavor chemistry (*1*). They had improved significantly the understanding of reactions occurring in cooking processes and would use this knowledge to create a new generation of savory meat flavors. The first commercial process reaction flavors were launched in the mid 1960's by Unilever through their subsidiary company Food Industries Ltd, later to become Quest and subsequently Givaudan. Prior to this, savory character and meaty notes were enhanced through

In Controlling Maillard Pathways To Generate Flavors; Mottram, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

the use of hydrolyzed vegetable proteins, autolyzates, monosodium glutamate, spice blends and meat extracts. These materials did not possess true meat flavor but were used to extend savory character present in food products.

Unilever began their research program at the Colworth House Research Laboratories in 1951 (*2*) at a time when, following the Second World War, a shortage of protein was causing national and international concern and research was being undertaken into the development of edible meat-like structures from texturized and spun proteins derived from groundnut and soy protein (*3*). These imitation meat products, or meat analogues as they are now known, had to be flavored and this provided the early impetus for research into meat flavor.

The meat flavor research at Colworth followed two different approaches; the first was to isolate and identify the aroma arising from beef on cooking and the second was to isolate and characterize the flavor precursors giving rise to beef flavor. The first approach did not produce any meaningful results for many years because the analytical techniques available then were not sensitive enough to detect the very low odor threshold sulfur compounds responsible for meat flavor. However, the second approach, the precursor approach, led to some important discoveries that laid the foundation of process flavor technology. In summary, what they found at Colworth was that when raw beef was extracted with cold water and dialyzed, the fraction having a molecular weight of less than 200 produced beef flavor on heating. This dialyzate was analyzed by paper chromatography and was found to contain about thirty two amino acids and small peptides, glucosamine, and three sugars, glucose, fructose and ribose. Following cooking of this dialyzate, paper chromatography revealed that two compounds had completely disappeared; the reducing sugar, ribose and the amino acid, cysteine. When these two compounds were heated together in water, a recognizable beef flavor was obtained. The resulting patent widened the discovery to include other pentose sugars, xylose and arabinose, hexose sugars such as glucose, other amino acids such as glutamic acid, glycine, alanine, peptides, such as glutathione, and protein hydrolyzates, which provided more complex amino acid profiles.

There followed then what might be called the era of serendipity where work on meat precursor chemistry led to the publication of a large number of patents. The approach was one of 'trial and error' or serendipity, because the compounds responsible for meat flavor had not been identified and, hence, the mechanisms by which they were formed, and the precursors responsible could only be surmised. Examples of some of the discoveries made during this period are outlined in Table I.

Clues about the compounds responsible for meat flavor were obtained from the study of model reactions and, in 1976, workers from International Flavors and Fragrances (IFF) (*4*) reported the presence of furans substituted in the three position with sulfur from a reaction involving thiamine hydrochloride, cysteine hydrochloride and hydrolyzed vegetable protein. Among the compounds formed was a thiol, 2-methyl-3-furanthiol (I) and its related disulfide (II) which had been patented by Evers prior to publication (*5*). It wasn't until 1986 that 2-methyl-3-(methylthio)furan (III) was found in cooked beef (*6*) and two years after that when the compounds found by the IFF team in model reactions, 2-methyl-3-furanthiol (I) and *bis*-(2-methyl-3-furyl)disulfide (II), were also found in cooked beef (*7*). The

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Table I. Serendipity Discoveries

Precursor Compounds	*Flavoring Effect*
The reaction of cysteine, xylose and hydrolyzed vegetable proteins. (The early Unilever patent)	Meaty flavors, both light and dark meat flavors are produced depending on the flavor character of the hydrolyzate used.
Arabinose	Found to be useful for light meaty character such as pork
Glucose and fructose	Useful for increasing the roast character of reaction flavors.
Methionine	Leads to the production of vegetable/potato character and can be used in process flavors to create stew, casserole and bouillon character.
Thiamine (Vitamin B1)	Produces beefy, meaty character.
Fats	Determines the species character of meat.
Hens eggs	Useful in making chicken flavors.
Yeast extracts	Provide and modify meaty character and meaty taste.
Cysteine/furaneol reaction	Gives chicken breast meat character
Cysteine/ascorbic acid reaction	Chicken, turkey and tuna flavor character.

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latter compound is reported to possess a rich aged-beef, prime rib aroma and this and the other furan thiols play an important role as character impact compounds of meat flavor. It had taken 28 years from the original patent filed by Unilever to the identification of the presence of these character impact compounds in meat. *Bis*-(2-methyl-3-furyl)disulfide (II) was found to have one of the lowest odor threshold levels ever recorded for a flavoring substance measured at 0.00002 µg/kg (ppb) in water. It is therefore not surprising that it took so long to find it in meat.

Reaction Pathways to the Furanthiols

Flavor chemists were interested in the mechanisms leading to the formation of the furanthiols and how these reactions could be used to maximize the formation of character impact compounds in process reaction flavors. Van den Ouweland and Peer (*8*), working for Unilever in the Netherlands, reported that the reaction between 4-hydroxy-5-methyl-3(2*H*)-furanone (norfuraneol) and hydrogen sulfide led to the formation of 2-methyl-3-furanthiol. Norfuraneol is formed from pentose sugars in the Maillard reaction by the mechanism shown in Figure 2. Whitfield and Mottram (*9*) subsequently showed that 2-methyl-3-furanthiol is formed from the reaction between norfuraneol and cysteine.

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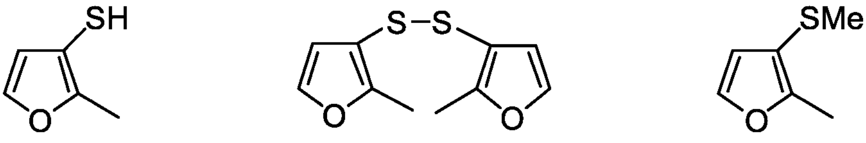

(I) (II) (III)

Figure 1. Character impact compounds of meat flavor

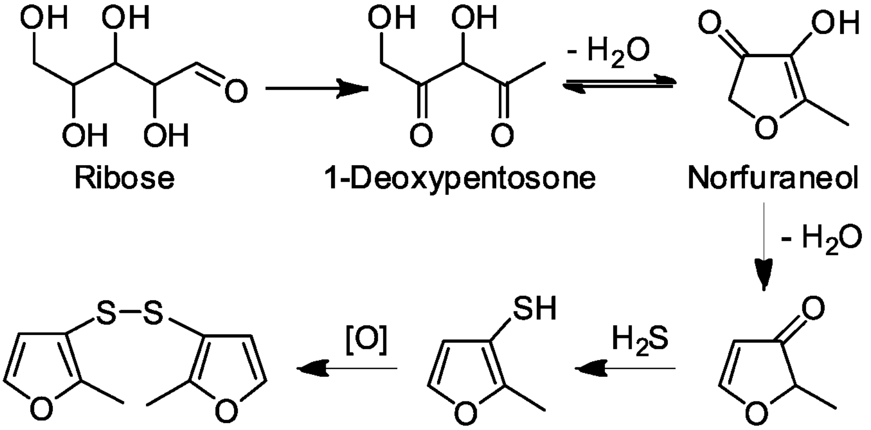

Figure 2. The formation of 2-methyl-3-furanthiol from pentose sugars

A second reaction route to the furanthiols was identified when Van der Linde et al (*10*) demonstrated the formation of 2-methyl-3-furanthiol from the heat degradation of thiamine. This represents one of the most amazing co-incidences of flavor chemistry, that an important character impact compound of cooked meat could be formed from two totally independent sources existing in raw meat. The mechanism from thiamine is shown in Figure 3.

Hofmann and Schieberle (*11*) added to our knowledge by showing that 2-methyl-3-furanthiol can be formed from hexose sugars, as well as pentose sugars, when heated in the presence of cysteine. In the reaction between ribose and cysteine, 2-methyl-3-furanthiol was preferentially formed under aqueous conditions at pH 3.0 but in the reaction between glucose and cysteine, its formation peaked at pH 5.0 and was produced in greater amounts when the two compounds were dry heated for 6 minutes at 180 °C. This led the authors to conclude that a different reaction pathway was operating. They proposed an alternative route to 2-methyl-3-furanthiol from the breakdown of glucose into fragments that included the precursors mercaptopropan-2-one and hydroxyacetaldehyde as shown in Figure 4. A third reaction route to 2-methyl-3-furanthiol had been uncovered.

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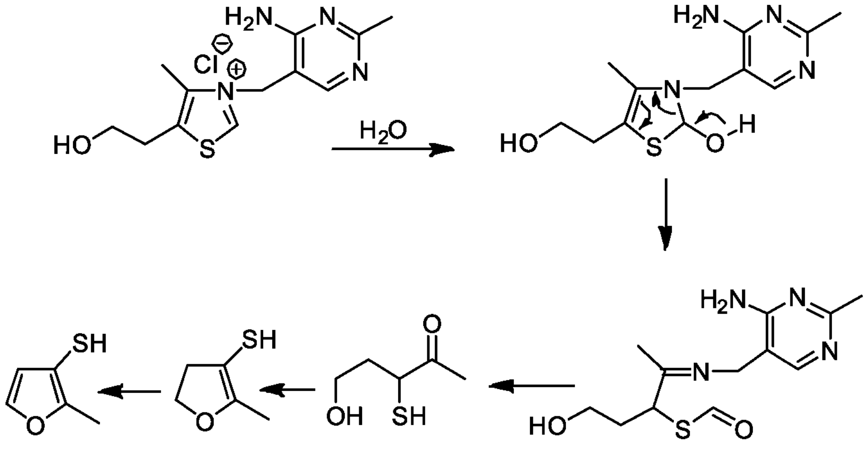

Figure 3. The formation of 2-methyl-3-furanthiol from thiamine

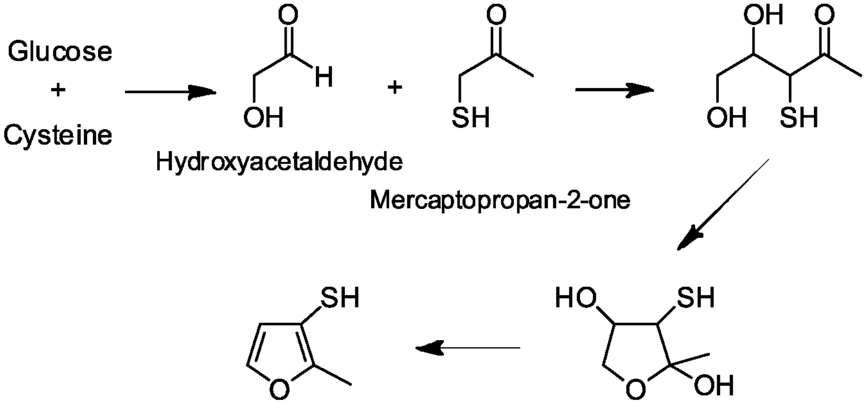

Figure 4. The formation of 2-methyl-3-furanthiol from hexose sugars

The Reaction of Ascorbic Acid with Cysteine

The reaction of ascorbic acid and isoascorbic acid with cysteine was patented in 1964 by General Mills (*12*). The reaction was found to produce flavors reminiscent of chicken, turkey and tuna and, although it has been used by flavorists to create these notes in process reaction flavors for over forty years, its chemistry has never been investigated. This undiscovered piece of serendipity from the early days of reaction flavor chemistry was investigated in our laboratories. Cysteine and ascorbic acid were reacted together in a buffered aqueous solution (pH 5.5) sealed in airtight PTFE-lined screw top glass reagent bottles at 140 °C for 30 minutes. The resulting mixture had a sweet, sulfurous aroma with undertones of raw chicken and chicken breast character and a taste (2% in warm water) of chicken breast, raw chicken and tuna steak. The headspace volatiles were collected using Solid Phase MicroExtraction (SPME) and Dynamic Headspace (DH) onto Tenax and analyzed by gas chromatography/mass spectrometry. A total of 203 compounds were found in the SPME extract and these surprisingly included both 2-methyl-3-furanthiol (I) and *bis*-(2-methyl-3-furyl)disulfide (II). The DH extract showed the presence of a large number of disulfides. When this was compared to an equivalent reaction between ribose and cysteine the number and concentration of disulfides was much lower in the ribose/cysteine system. Ascorbic acid is easily oxidized to dehydro-L-ascorbic acid (*13*), which in turn oxidizes thiols, formed in the reaction with cysteine, to disulfides reverting back to ascorbic acid in the process. This possibly explains the relatively high quantities of disulfides in the reaction between cysteine and ascorbic acid. It also provides a useful tool for the flavorist to use for the generation of disulfides in process reaction flavors, increasing their strength and enhancing their character.

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Conclusions

The fact that 2-methyl-3-furanthiol can be formed from both pentose and hexose sugars and two vitamins occurring in meat, ascorbic acid and thiamine, must represent one of the most amazing co-incidences of flavor chemistry. The reaction pathways are summarized in Figure 5. These reaction pathways leading to important character impact compounds of meat provide scope for the creative flavorist to produce high impact reaction flavors and the flexibility to generate different meat flavor notes.

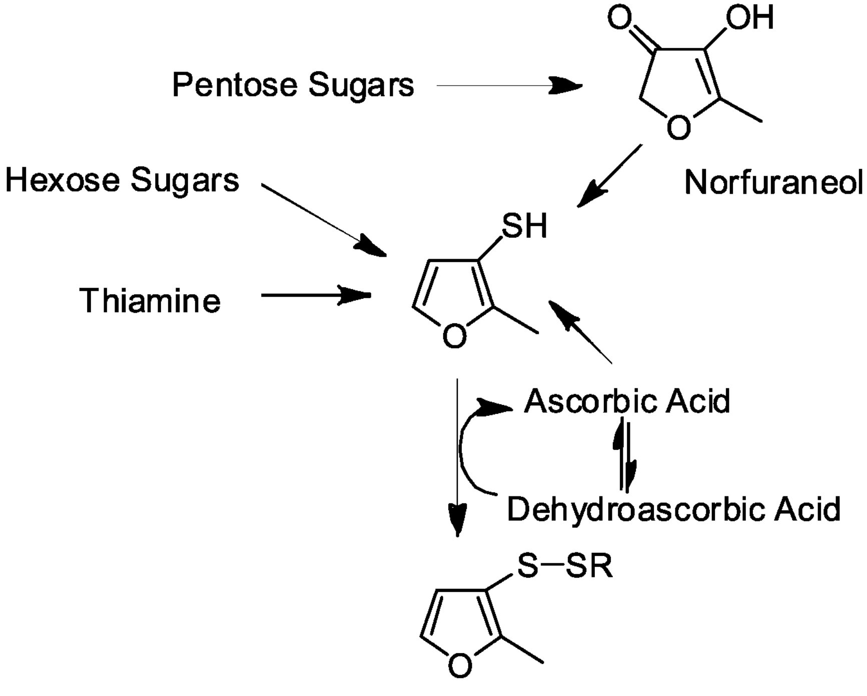

Figure 5. Pathways to 2-methyl-3-furanthiol

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Chapter 8

Meat Flavor Generation in Complex Maillard Model Systems

Reactants Synergetic and/or Competition Effect?

S. I. F. S. Martins,[*] A. Leussink, E. A. E. Rosing, G. A. Desclaux, and C. Boucon

**Unilever R&D Vlaardingen, Olivier van Noortlaan 120, P.O. Box 114,
3130 AC Vlaardingen, The Netherlands
[*]sara.martins@unilever.com**

Formation of non-sulfur compounds (4-hydroxy-5-methyl-3(2*H*)-furanone, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 5-hydroxymethylfurfural, 2-furfural) and sulfur compounds (2-methyl-3-furanthiol, 2-furfurylthiol) that contribute to meat flavor were investigated in Maillard complex model systems containing mixtures of sugars and amino acids. Customized fractional factorial design was used to select the composition of the systems. Volatiles were quantified by GC-MS and responses analyzed by regression analysis. Significant ($p<0.05$) synergetic and/or competition effects were observed between reactants. The presence of more than one sugar and/or amino acid had a clear impact on the formation of key meat volatiles as well as on the mixture's final sensory profile. Preliminary insight on the Maillard reaction mechanism in complex mixtures as they occur in real food products is given.

Introduction

The aroma of cooked meat is provided by a complex mixture of volatile compounds produced during cooking (*1*, *2*), where sulfur-containing compounds are considered to be particularly important. During cooking, a major route to these compounds is the Maillard reaction between reducing sugars and the amino acid cysteine. One of the most important sugars present in meat is ribose. The

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precursors, ribose and cysteine, are consequently employed in the production of process flavors, but ribose is frequently replaced by its less expensive isomer xylose (*3*, *4*). Numerous model reactions with ribose and cysteine have been carried out to study meat flavor generation (*3–7*). The thiols 2-methyl-3-furanthiol (MFT) and 2-furfurylthiol (FFT) are amongst the most important aroma impact compounds formed during the thermal reaction of ribose and cysteine. These molecules are also found in cooked meat (*8*, *9*), as well as in commercial meat flavorings (*10*) and contribute significantly to their aroma.

The sulfur-containing volatiles are likely to be formed from the reactions of hydrogen sulfide with carbonyl compounds. Hydrogen sulfide can derive from the hydrolysis of cysteine or from the Strecker degradation of cysteine in the presence of dicarbonyl compounds. Carbonyl and dicarbonyl compounds derive mainly from the breakdown of the reducing sugars via the Maillard reaction. In particular for pentoses, two important intermediates are 4-hydroxy-5-methyl-3(2*H*)-furanone (also known as norfuraneol) and 2-furfural. The initial step of the Maillard reaction produces an Amadori compound, which is deaminated and dehydrated via either 1,2-enolisation or 2,3-enolisation (Figure 1). For pentoses, the 1,2-enolization route gives 2-furfural via 3-deoxypentosone, whereas 2,3-enolization results in 1-deoxypentosone and 4-hydroxy-5-methyl-3(2*H*)-furanone. Model experiments with 2-furfural and hydrogen sulfide showed this intermediate as important for the formation of FFT (*10*) whereas model experiments with 4-hydroxy-5-methyl-3(2*H*)-furanone with cysteine or hydrogen sulfide showed this intermediate as important for the formation of MFT (*11*).

Meat flavor is a vast field of research where many studies have been conducted (*1–14*). However, the knowledge has mainly been built in simple model systems (one sugar and one amino acid), which do not always correspond to what is actually formed in current recipes. Therefore, in the present study complex Maillard model systems were examined. The selection of the reactants was based on their ability to generate meat flavor and/or meat flavor intermediates, like xylose, cysteine and 5-ketogluconic acid, as well as on their abundance in food products, like glucose, glycine and glutamic acid. Statistical modeling was applied to identify important factors and significant ($p<0.05$) synergetic and/or competition effects between the reaction precursors with impact on the formation of key meat volatiles and on the final sensory profile.

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Experimental Procedures

Sample Preparation

Samples were heated in an oil bath at 100°C in pyrophosphate buffer solution (50 mL, 0.2M, pH 6). Xylose and cysteine were the expected two main precursors in meat flavor generation; their minimal concentration was therefore 0.05%. The selection of other reactants, glucose, glycine and glutamic acid was based on their abundance in food products. Besides these, a potential alternative ingredient to xylose, 5-ketogluconic acid was also studied (*15*). D-Glucose, D-Xylose, L-Cysteine, L-Glutamic acid and L-Glycine, were purchased from Sigma-Aldrich (Steinheim, Germany); 5-Ketogluconic acid was donated by a supplier.

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Experimental Design Definition

Complex model systems (more than one sugar and amino acid) were studied using a statistical experimental design. Seven variables were included in this study as summarized in Table I. Six ingredients at 2 levels (absent/present or low/high); reaction time at 3 levels; constant pH6 and temperature at 100°C. A D-optimal customized fractional factorial design with 32 combinations was selected. In theory, this design allows the estimation of the main effect of the ingredients and reaction time, as well as 2 factor interactions and the quadratic effect of time. The design was further divided in 4 blocks of 8 samples to fit sample preparation and measurements constraints.

Modeling by Regression Analysis

The results for each response were analyzed using Ordinary Least Square regression. For each response a regression model was included. It comprised the main effect of each factor, quadratic effect of time and 2-way interactions between all pairs of ingredients. Effects that were not found significant nor contributing to any interaction were removed and a new, simplified model was estimated. Due to heteroscedasticity, the log transformed data ($\log_{10}$) was analyzed. Null values were replaced by the lowest measured values. The mean value for each experiment was used as a response. The analyses were performed using JMP-v8 software. Effects were deemed significant at alpha=0.05. Modeling results are summarized in Tables II to IV, indicating for each compound the significant effects and their relative importance. A positive effect or interaction (synergy between two components) is indicated with a positive sign (+) whereas a negative effect or interaction (competition between components) is indicated with a negative sign (–). The number of signs indicates the strength of the effect (e.g. +++ indicates a strong positive effect whereas + indicates a weaker, although both show significant effect). The quality of the model was indicated using two statistics, namely the R-square statistics and the root mean square error (RMSE). The R-square reports the proportion of information captured by the model. It takes values between 0 and 1, 1 representing a perfect fit. The RMSE gives an indication of the average error made on predictions.

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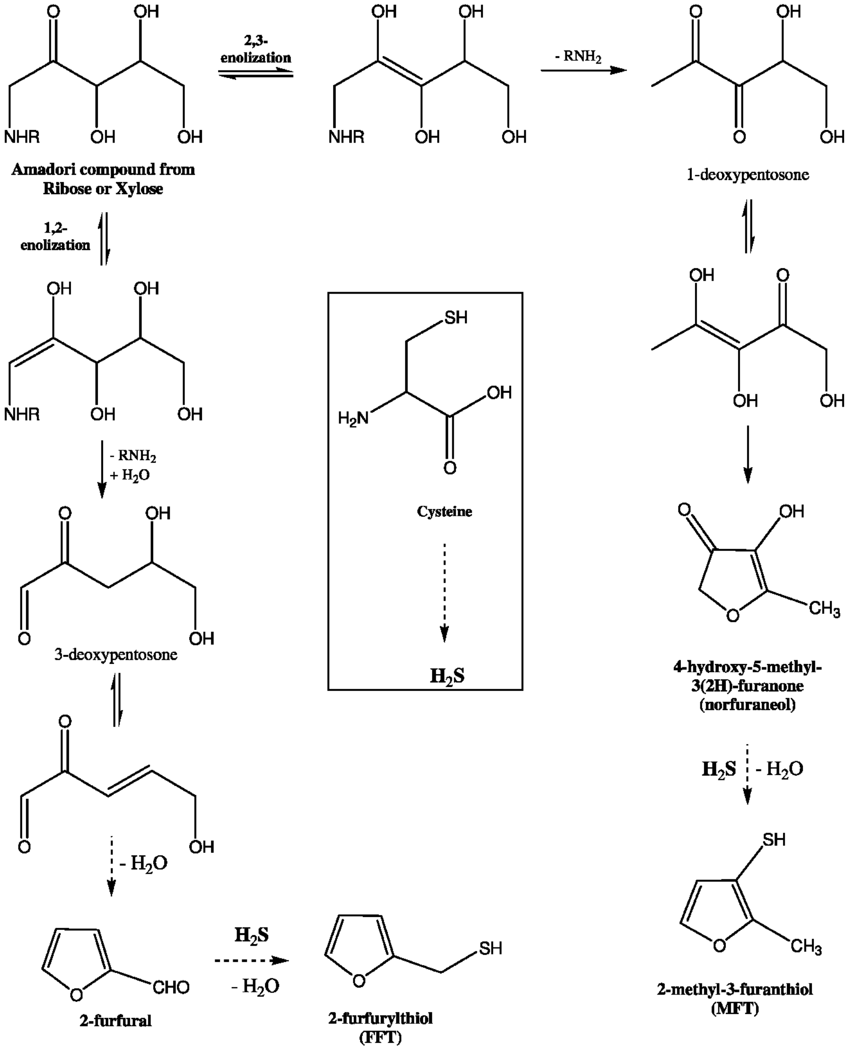
Amadori compound from Ribose or Xylose
2,3-enolization
- RNH2
1-deoxypentosone
1,2-enolization
- RNH2 + H2O
3-deoxypentosone
Cysteine
H2S
4-hydroxy-5-methyl-3(2H)-furanone (norfuraneol)
H2S - H2O
- H2O
H2S
- H2O
2-furfural
2-furfurylthiol (FFT)
2-methyl-3-furanthiol (MFT)
NHR
OH
O
SH
H2N
CHO
CH3

Figure 1. Formation of 2-methyl-3-furanthiol (MFT) and 2-furfurylthiol (FFT) from H_2S and pentose derived intermediates 4-hydroxy-5-methyl-3(2H)-furanone and 2-furfural, respectively, formed via the Amadori compound in the Maillard reaction.

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Table I. Factors and levels selected (the table also gives an overview of the responses measured and their respective minimum and maximum levels)[a]

	Name	*Lower Level*	*Higher Level*
Factors	D-Xylose (%)	0.05	3
	D-Glucose (%)	0	3.6
	5-ketogluconic acid (%)	0	3.0
	L-Cysteine (%)	0.05	0.75
	L-Glycine (%)	0	1.5
	L-Glutamic acid (%)	0	1.5
	Time (h)	0	3.0
Responses	MFT (ppm)	0	348.4
	FFT (ppm)	0	352.4
	Norfuraneol (ppm)	0	10003.1
	5-HMF (ppm)	0	1.6
	2-Furfural (ppm)	0	8.9
	Furaneol (ppm)	0	42.3

[a] 2-methyl-3-furanthiol (MFT), 2-furfurylthiol (FFT), 4-hydroxy-5-methyl-3(2*H*)-furanone (norfuraneol); 5-hydroxymethylfurfural (5-HMF); 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol)

Quantification of Sulfur Compounds

The quantification of 2-methyl-3-furanthiol (MFT) and furfurylthiol (FFT) can be quite difficult due to their instability, in particular to their ability to oxidize into disulfides (*16*). In literature most methods use extraction with organic solvents. As alternative to extraction, the present methodology using derivatization with DDT was developed, allowing the quantification of the targeted thiols in food matrices of different composition, which may include fat for instance.

2-Methyl-3-furanthiol (MFT), 2-furfurylthiol (FFT) and 1,4-dithioerythritol (DTT) were purchased from Sigma-Aldrich (Steinheim, Germany). [$^2H_3$]-2-methylfuran-3-thiol (MFT*) and [$^2H_2$]-furfurylthiol (FFT*) were purchased from aromaLAB AG (Planegg-Martinsried, Germany). DTT (2%) and stable isotope labeled internal standards MFT* (12 ng/g) and FFT* (21 ng/g) were added to the sample to be analyzed (5 g) in a 10 mL vial with screw cap. The mixture was then homogenized for 1 min using a vortex at 1000 rpm. MFT and FFT linearity in pyrophosphate buffer, containing 2% DTT, was determined from 0.5 to 800 ng/g relative to its labeled internal standard. Analyses were performed on an Agilent 6890 gas chromatograph (GC) with a 5973 mass spectrometric detector (MSD). Headspace SPME was performed using a Gerstel MPS-2 autosampler (Mulheim, Germany). All samples were stored in a cooled autosampler tray (5°C). The sample was pre-heated for 2 mins at 60°C before the extraction was done with a 100µm PDMS fiber at 60°C for 30 mins, with 250 rpm agitation. The fiber was then desorbed in a split/splitless injector, operated in splitless mode with a Restek SPME liner, for 65 seconds at 250°C. Finally the fiber was baked out in a needle heater for 20 mins at 250°C. Helium carrier gas (1 mL/min constant flow) was used through a Restek Rxi-5MS capillary column (30 m x 0.25 mm i.d. with 0.25 µm film thickness). The oven temperature was held for 2 min at 0°C and then raised to 75°C at 3°C/min followed by a 30°C/min increase to 250°C held for 15 min. The MSD was operated in single ion monitoring mode. The molecular ion of each compound, with qualifier ions, was used for quantification.

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Quantification Non-Sulfur Compounds

4-Hydroxy-5-methyl-3(2*H*)-furanone (norfuraneol), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (Furaneol, a registered trademark of Firmenich S.A.), 2-furfural and 5-hydroxymethylfurfural were all purchased from Sigma-Aldrich (Steinheim, Germany). Analyses were performed on an Agilent 6890 gas chromatograph (GC) with a 5973 MSD. Liquid injection was performed using a Gerstel MPS-2 autosampler (Mulheim, Germany). The liquid samples were obtained by extraction. To 2 mL of the sample mixture, 1 mL saturated NaCl solution was added and extracted with 1 mL ethylacetate using a vortex during 1 min at 2500 rpm. One microliter of the ethylacetate layer was injected into a Cooled Injection System (CIS) at 80°C. After 0.2 min equilibration time, the CIS heated up at 10°C/sec to 250°C. The injector was operated in the splitless mode except for norfuraneol (split 1:200). Helium carrier gas (1 mL/min constant flow) was used through a Wax 58 capillary column (25 m x 0.15 mm i.d. with 0.25 film thickness). The oven temperature was held for 2 min at 60°C and then increased with 10°C/min to 240°C for 9 min. The molecular ion of each compound, with qualifier ions, was used for quantification. The quantification was done by external calibration.

Sensory Evaluation

A panel of experts performed the evaluation. The attributes selection was done in consensus by smell, using samples from the experimental design. The data was categorized according to six categories from 0 (blank) – absent smell to 5 – high level of smell. Multivariate analysis, including correlation analysis and principal components analysis (with Varimax rotation) was applied to the data, which were treated as continuous. Principal component analysis was performed on the sensory data only and subsequently on the dataset including both smell impressions and ingredients as variables.

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Results and Discussion

The ability of Maillard complex model systems to generate meat flavor was investigated under cooking conditions. Similar to the decomposition of xylose (Figure 1), the degradation pathways of glucose were expected to proceed via 1,2-enolisation forming 5-hydroxymethylfurfural and via 2,3-enolisation forming furaneol. 5-Ketogluconic acid as an alternative to pentoses in meat reaction flavors production was expected to yield norfuraneol as the main product (*15*). The analysis of the results was done first by understanding the impact of the reactants on the key non-sulfur meat flavor intermediates and after on the formation of the thiols 2-furfurylthiol and 2-methyl-3-furanthiol. The impact on the sensory profile will be discussed last.

Linking Modeling Results to Maillard Chemistry

Formation of Key Non-Sulfur Compounds Related to C_6 Sugars

5-HMF and furaneol are two important intermediates in the Maillard reaction involving hexoses degradation, such as glucose. The modeling results are shown in Table II and III, respectively.

Table II. Main factors in 5-HMF formation[a,*]

Xyl	*Glc*	*Cys*	*Xyl*Glu*	*Glc*Gly*	*Cys*Glu*	*R^2*	*RMSE*
+++	++	---	++	-	--	0.86	0.11

[a] Xylose (Xyl); Glucose (Glc); Glycine (Gly); Cysteine (Cys); Glutamic acid (Glu); R-square statistics (R^2); root mean square error (RMSE). * Main factors as significant & relative importance. (+) positive effect or interaction (synergy between two components); (-) negative effect or interaction (competition between components). The number of signs indicates the strength of the effect.

Table III. Main factors in Furaneol formation[a,*]

Gly	*Time*	*Xyl*Glc*	*R^2*	*RMSE*
+	+++	-	0.82	0.78

[a] Xylose (Xyl); Glucose (Glc); Glycine (Gly); Cysteine (Cys); Glutamic acid (Glu); R-square statistics (R^2); root mean square error (RMSE). * Main factors as significant & relative importance. (+) positive effect or interaction (synergy between two components); (-) negative effect or interaction (competition between components). The number of signs indicates the strength of the effect.

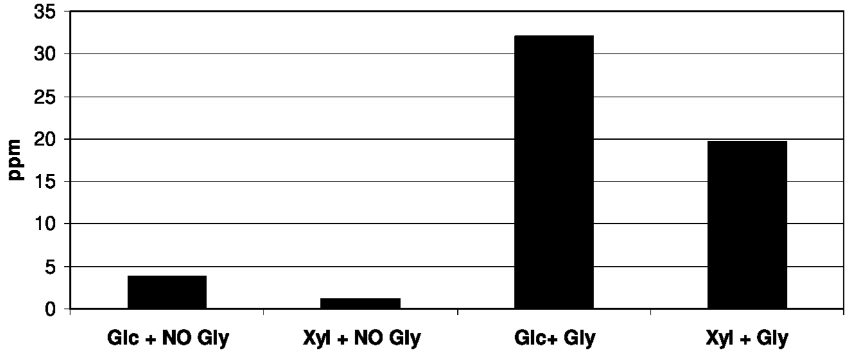

Figure 2. Furaneol formation in reducing sugar and amino acids mixtures, with and without glycine (Gly) after 3h of heating at 100°C, pH 6. Reactants concentration 20 mmol/100mL. Glucose (Glc),Xylose (Xyl).

Even though a C_6 compound, the formation of 5-HMF was significantly dependent on the presence of xylose. In particular a positive interaction between xylose and glutamic acid in 5-HMF generation was observed. (Table II). No significant effect of glycine was observed in 5-HMF formation. However, in the presence of both glucose and glycine a negative effect was detected, an indication that these are probably not the preferred reactants for 5-HMF formation. Also, cysteine showed a clear negative effect on 5-HMF formation. Regarding furaneol, the presence of glycine showed a clear positive effect, as well as the reaction time (Table III). Looking at the experimental results, furaneol was generated in both glucose and xylose systems, which was clearly enhanced in the presence of glycine (Figure 2), in line with the modeling results. Between xylose and glucose a negative interaction was observed, indicating that when both sugars are present in the reaction mixture, they compete in furaneol formation (Table III).

Xylose is a C_5 sugar and in order to generate C_6 intermediates, like 5-HMF and furaneol, an extra source of carbon would be required. Two possible routes would be either by rearrangement after cleavage of the carbohydrate skeleton or by reaction with amino acids degradation products. Using labeling techniques, Blank et al. (*17*) showed that the amino acids can, in fact, participate in the formation of furanones from pentoses through the Strecker degradation. In the presence of glycine, furaneol was shown to be formed from xylose where the C_5 structure remained intact and the C_1 unit came from the formaldehyde, the Strecker aldehyde from glycine. This mechanism supports the current modeling results, where 5-HMF and furaneol formation were related significantly to the reaction between xylose and glutamic acid and xylose and glycine, respectively.

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Formation of Non-Sulfur Compounds Related to C_5 Sugars

4-Hydroxy-5-methyl-3(2*H*)-furanone (norfuraneol) and 2-furfural are two important intermediates in meat flavor generation via the Maillard reaction, involving pentoses. Their modeling results are presented in Table IV and V, respectively.

Xylose was clearly related to the formation of both norfuraneol and 2-furfural, in line with the mechanism presented in Figure 1. However, glucose showed no significant effect on the formation of either of these compounds. Also, in glucose reaction mixtures (i.e. with no KGA and xylose at 0.05%) norfuraneol and 2-furfural were formed in very low amounts. This discrepancy between hexoses and pentoses in norfuraneol and 2-furfural formation has also been reported in literature (*14*). A clear and significant positive effect of KGA in norfuraneol formation was observed (Table IV). As the modeling results show, and as anticipated by De Rooi et al. (*15*), KGA could be a suitable alternative to pentoses in norfuraneol formation. However, between xylose and KGA a negative interaction was observed. When xylose is present in the reaction mixture, the impact of KGA on norfuraneol formation is reduced (Figure 3). Both glycine and cysteine showed a clear negative effect on 2-furfural formation, which for glycine became positive in the presence of glucose. No plausible mechanism can be suggested at this stage. Further investigations would be required.

Table IV. Main factors in Norfuraneol formation[a,*]

Xyl	*KGA*	*Gly*	*Time*	*Xyl*KGA*	*Cys*Glu*	*R^2*	*RMSE*
++	++	++	++	-	-	0.85	0.42

[a] Xylose (Xyl); 5-ketogluconic acid (KGA); Glucose (Glc); Glycine (Gly); Cysteine (Cys); Glutamic acid (Glu); R-square statistics (R^2); root mean square error (RMSE). * Main factors as significant & relative importance. (+) positive effect or interaction (synergy between two components); (-) negative effect or interaction (competition between components). The number of signs indicates the strength of the effect.

Table V. Main factors in 2-Furfural formation[a,*]

Xyl	*KGA*	*Gly*	*Cys*	*Time*	*Glc*Gly*	*R^2*	*RMSE*
+++	++	-	--	++	+	0.79	0.43

[a] Xylose (Xyl); 5-ketogluconic acid (KGA); Glucose (Glc); Glycine (Gly); Cysteine (Cys); Glutamic acid (Glu); R-square statistics (R^2); root mean square error (RMSE). * Main factors as significant & relative importance. (+) positive effect or interaction (synergy between two components); (-) negative effect or interaction (competition between components). The number of signs indicates the strength of the effect.

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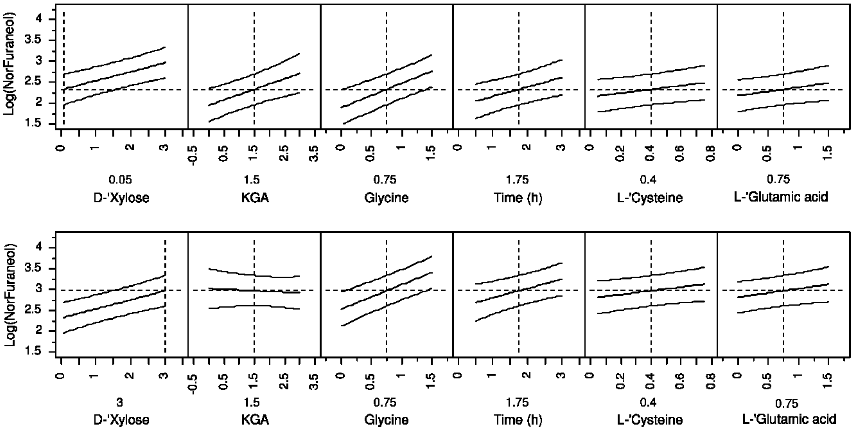

Figure 3. Modeling results showing the interaction between xylose and KGA levels on norfuraneol formation. At low level of xylose (0.05%) positive effect of KGA, whereas at high level of xylose (3%), the effect of KGA disappears.

Major Observations on Formation of Key Non-Sulfur Meat Flavor Compounds

As expected the two C_5 related key non-sulfur compounds, norfuraneol and 2-furfural, are mainly formed in the presence of xylose and KGA. Glucose showed no significant effect in their formation. Xylose, on the other hand, seems to be involved in the formation of the C_6 related key non-sulfur compounds, furaneol and 5-HMF. According to the modeling results, and in line with what has been reported in literature (*17*), the underlying mechanism seems to imply that the reaction with the Strecker aldehyde from the amino acids occurred. Moreover, sugars and sugar related compounds, like KGA, do not show a synergetic effect between them. Xylose and KGA competed in norfuraneol formation, as well as glucose and xylose competed in furaneol formation.

Regarding the amino acids impact on the reaction mechanism, glutamic acid seemed to be involved in the formation of 5-HMF when xylose was present. However, on its own no major significant effect was observed for the formation of non-sulfur compounds. On the contrary, glycine showed a clear positive effect on both furaneol and norfuraneol formation. Also, cysteine showed a clear significant effect, even if negative, in both 5-HMF and 2-furfural formation. These results seem to give some indication that cysteine inhibits sugar degradation via the 1,2-enolisation step, whereas glycine is mainly involved in sugars degradation via the 2,3-enolisation step by promoting it.

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Key Sulfur Meat Flavor Compounds Formation.

2-Methyl-3-furanthiol (MFT) and 2-furfurylthiol (FFT) are among the most important aroma impact compounds present in meat flavor. The modeling results are presented in Table VI and VII, respectively.

Table VI. Main factors in MFT formation[a,*]

Cys	*Gly*	*Time*	*Xyl*KGA*	*Xyl*Gly*	*Cys*Gly*	*R^2*	*RMSE*
++	++	++	-	-	+	0.82	0.52

[a] Xylose (Xyl); 5-ketogluconic acid (KGA); Glucose (Glc); Glycine (Gly); Cysteine (Cys); R-square statistics (R^2); root mean square error (RMSE). [*] Main factors as significant & relative importance. (+) positive effect or interaction (synergy between two components); (-) negative effect or interaction (competition between components). The number of signs indicates the strength of the effect.

Table VII. Main factors in FFT formation[a,*]

Xyl	*Cys*	*Gly*	*Time*	*Xyl*Gly*	*Cys*Gly*	*R^2*	*RMSE*
++	++	++	-	+	+	0.82	0.52

[a] Xylose (Xyl); 5-ketogluconic acid (KGA); Glucose (Glc); Glycine (Gly); Cysteine (Cys); R-square statistics (R^2); root mean square error (RMSE). [*] Main factors as significant & relative importance. (+) positive effect or interaction (synergy between two components); (-) negative effect or interaction (competition between components). The number of signs indicates the strength of the effect.

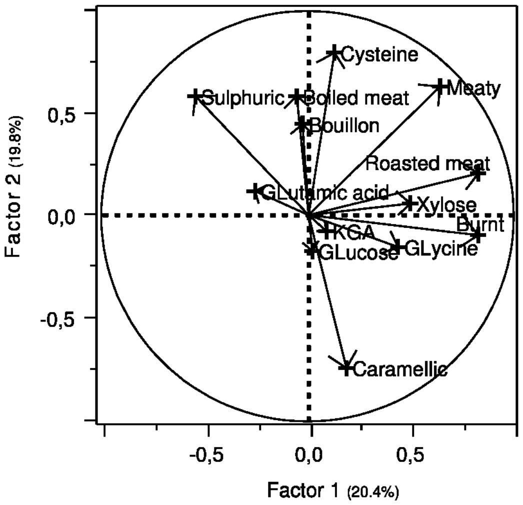

Figure 4. Principal components analysis on combined sensory and ingredients information.

Cysteine showed a clear positive effect on both MFT and FFT formation. This was, of course, expected, since it was the only reactant with a sulfur source. However, a clear positive interaction between cysteine and glycine was also observed. MFT formation was strongly enhanced in the presence of glycine. These results corroborate with the observation that glycine had also a clear positive effect on norfuraneol formation (Table IV). The two amino acids seem to complement each other. According to literature, the preferential generation of MFT from the pentoses is via norfuraneol as the intermediate (*14*, *18*). The assumption based on the modeling results is that glycine enhances norfuraneol formation via 2,3-enolisation which then reacts with hydrogen sulfide from cysteine to generate MFT. This is preliminary evidence and further studies are required to confirm this hypothesis. Contrary to glycine, glutamic acid showed no significant effect on MFT formation. The competition effect observed between cysteine and glutamic acid on norfuraneol formation (Table IV) may indicate a possible change in the reaction mechanism of MFT generation. Potential implications in the mixture final sensory profile may be expected.

Regarding FFT formation, xylose had a clear positive effect whereas the impact of glucose was minimal. The same was observed with MFT formation. This corroborates with the discrepancy observed between hexoses and pentoses on 2-furfural and norfuraneol formation, where the impact of glucose observed was also minimal. The effect of glucose was significant on the formation of furaneol and 5-HMF, two degradation products from hexoses via 2,3-enolisation and 1,2-enolisation, respectively. The involvement of these key intermediates, in either MFT or FFT formation, seems not to be relevant according to the modeling results, and this is in line with what has been reported in literature (*14*). Also, Shu et al. (*19*) identified the volatile formation from the reaction between cysteine and furaneol at different pHs. At pH 5.1 the volatiles were dominated by the dimethyl derivatives of 4-hydroxy-3(2*H*)-thiophenone and 1,2,4-trithiolane, although when pH increased to 7.1 pyrazines became the major products.

Analysis of the Sensory Results

The principal component analysis, illustrated in Figure 4, combines both sensory and ingredient information. The first dimension indicates an association between roasted meat and burnt notes and the presence of xylose and glycine whereas the second dimension indicates an association between sulphuric, boiled meat and bouillon notes and the presence of cysteine.

The meaty smell shows high loadings on both dimension 1 and 2 and it is correlated with the presence of xylose and cysteine. This confirms the data from literature where these precursors are commonly used in meat flavor generation. In the absence of cysteine the caramellic notes prevailed.

It should be noted that two dimensions do not seem to be enough to justify all the variability obtained in the studied Maillard systems. The first two principal components accounted for only 20.4% and 19.8% of the variability in the data, respectively. Due to the complexity of the systems more than two dimensions are required. The analysis of the sensory results should therefore be seen more as a qualitative indication than a quantitative one.

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Conclusions

All models showed a moderate to reasonable fit, where the R-squared values varied between 0.8 and 0.9. Significant synergetic and competition effects were observed between reactants in meat flavor generation via the Maillard reaction. Sugars clearly compete between themselves, whereas cysteine and glycine showed a clear synergetic effect with positive effect on MFT formation. A positive interaction was also observed between xylose and both glutamic acid and glycine in the formation of HMF and furaneol, respectively, highlighting the importance of the Strecker degradation within the Maillard reaction.

This study was intended as a screening experiment, indicating which ingredients have an effect on the formation on which compound, the direction of these effects and possible interactions. However, these findings would need further validation in subsequent experiments, especially if we were to predict compound formation with statistical exactitude.

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Chapter 9

Flavor Development in a Meat-Based Petfood Containing Added Glucose and Glycine

Jane K. Parker,*,[1] Dimitrios P. Balagiannis,[1] Neil Desforges,[2] and Donald S. Mottram[1]

[1]Department of Food and Nutritional Sciences, University of Reading, Whiteknights, Reading, RG6 6AP, United Kingdom
[2]Waltham Centre for Pet Nutrition, Waltham-on-the-Wold, Melton Mowbray, Leicestershire, LE14 4RS, United Kingdom
***j.k.parker@reading.ac.uk**

Glucose and glycine, as reactive precursors of the Maillard reaction, are important for the generation of flavour in many cooked foods. Although there have been many studies on generation of flavor in aqueous model systems, such systems are not always representative of real food products. In this study, glucose and glycine were systematically added to a complete petfood recipe and the changes in volatile profile gave a clear indication of the limiting precursors for the formation of 2-furfural, 2,5-dimethylpyrazine and trimethylpyrazine. Use of $^{13}C_6$ labeled glucose on a smaller scale confirmed many of the findings, and the principal formation pathways for each of these volatiles were established.

Introduction

The Maillard reaction has a critical role in the development of flavor during the cooking of food. The understanding and control of this reaction is therefore of great importance to the food industry in order to manipulate the flavor profile of their products. Many research groups have worked in this area over the last 60 years, producing well over 500 publications devoted to flavor formation alone and these have been comprehensively reviewed by Nursten (*1*). However, only a small proportion of these consider the Maillard reaction in a complete food matrix, and even fewer attempt to explain changes in the volatile profile in a quantitative and

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predictive manner. The aim of this work was to elucidate key reaction pathways in a complex food matrix which could be used as a basis for predictive mathematical modeling (*2*).

Many studies on the Maillard reaction have been carried out in aqueous model systems and some of these are reported in previous ACS symposia (*3–5*). Reactants were generally limited to one or two reducing sugars and one or two amino acids, and proposed pathways were often confirmed using stable isotope labeling of key precursors (*6–9*). More detailed kinetic studies of the reaction between glucose and glycine in aqueous solution have been carried out by Wedzicha (*10*), but these tend to focus on formation of color rather than flavor.

Some research groups have investigated the Maillard reaction in more complex matrices including, for example, phospholipids (*11*) or in complete food systems, particularly meat. Aliani (*12*) focussed on the generation of sulfur compounds in cooked chicken and confirmed the role of ribose as an important flavor precursor whereas Meinert *et al.* (*13*) and Young (*14*) looked at adding different carbohydrates to pork and sheepmeat respectively. Parker (*15*) compared the volatile profiles generated from beef liver (where the predominant sugar is not ribose but glucose) with beef muscle and showed that the pyrazine profile could be altered by addition of glycine.

The present study used a real petfood recipe as a standard base in which levels of glucose and glycine were manipulated simultaneously in the presence of cysteine. This paper focuses on some of the non-sulfur-containing components of the volatile profile.

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Materials and Methods

Preparation and Cooking of Standard Products in Cans

A standard chunks-in-jelly petfood containing approximately 10 % protein, 5 % fat, 82 % moisture and 2% ash was prepared from a single batch of chunks and a gravy mix. The ratio of chunks to gravy was approximately 1:1. The chunks had been prepared from a typical petfood meat based emulsion which had been subjected to mild thermal treatment typical of those used to prepare this type of petfood. The standard gravy mix was used to manipulate the concentration of glucose and glycine prior to processing. Both glucose and glycine were added to the gravy to give three levels in the final product (L0, L1 and L2) containing 0, 5 and 20 mmol/100g of added glucose or glycine, respectively. Taking account of the levels already present in the chunk, this gave glycine levels of 25, 400 and 1525 mg/100 g and glucose levels of 150, 1050 and 3750 mg/100 g. All combinations of these precursor levels gave a total of nine products. All products were cooked in standard 400 g cans in a laboratory-scale sterilizer (Barriquand Steriflow 1311 EL) using a typical canning process at 125°C.

Preparation and Cooking of ^{13}C Labeled Products in Ampoules

A second batch of chunks (500 g), with similar composition to those used for the standard canned products, was mixed with an equal quantity of deionized water and homogenized in a domestic food processor (Megamix Cuisine Système 5100) for 1 min. The slurry was then centrifuged for 20 min at 29800*g* at 4°C and the supernatant filtered (Whatman filter no. 3) under vacuum. L-Methionine (1 mmol), L-cysteine (1 mmol) and glycine (10 mmol) were added to 200 mL of the filtered extract and the resulting extract divided into two equal aliquots (100 mL). D(+)-Glucose (5 mmol) was added to one portion and $^{13}C_6$ D(+)-glucose (Isotec, 99% ^{13}C) (5 mmol) was added to the other portion. The concentrations of added glucose and glycine in the final extracts were the same as L1 for the standard products (5 mmol) and after accounting for endogenous glucose, the proportion of the total glucose which was labeled with $^{13}C_6$ was 80%. Aliquots of each extract (20 mL) were sealed in 30 mL glass ampoules, immersed in an oil bath at 130 °C and heated for 1 h using the method described by Balagiannis *et al.* (*2*). After heating, each ampoule was immersed in a dry ice/methanol mixture at -50 °C to stop the reaction.

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Analysis of Volatiles

For analysis of the standard petfood in cans, the contents of one can of petfood were homogenized for 30 seconds in a domestic food processor (Megamix Cuisine Système 5100) and 25 g was transferred to a 250-mL conical flask with a screw-thread. For analysis of the extracts processed in glass ampoules, the extracts were diluted either 1:9 or 1:1 with HPLC grade water and 10 g was transferred to a 250-mL conical flask. In both cases, nitrogen, at 40 mL/min was passed over the extract, which was held at 60 °C for 60 min, sweeping volatiles onto a glass-lined, stainless steel trap (105 mm × 3 mm i.d.) containing 85 mg Tenax TA (Scientific Glass Engineering Ltd., Milton Keynes, UK). A standard (100 ng 1,2-dichlorobenzene in 1 µL methanol) was added to the trap at the end of the collection, and excess solvent and any water retained on the trap were removed by purging the trap with nitrogen at 100 mL/min for 10 min. Extractions were performed in triplicate.

All GC-MS analyses were performed on a Hewlett Packard 5972 mass spectrometer, coupled to an HP5890 Series II gas chromatograph and a G1034C Chemstation. A CHIS injection port (Scientific Glass Engineering Ltd.) was used to thermally desorb the volatiles from the Tenax trap onto the front of a CPSil 8 fused silica capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness; Varian, UK). The extracts prepared in the glass ampoules were also analyzed on a DB-Wax column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific). In both cases, the front of the column was made into a coil of five loops, which were cooled in solid carbon dioxide, contained within a 250-mL beaker, during the desorption period of 5 min, while the oven was held at 40°C. After desorption, the temperature of the oven was held at 40°C for a further 2 min, before being raised at 4 °C/min to 280 °C. Helium at 20 psi was used as the carrier gas, resulting in a flow of 1.2 mL/min at 40°C. A series of *n*-alkanes (C_5-C_{25}) in diethyl ether was analyzed, under the same conditions, to obtain linear retention index (LRI) values for the aroma components.

The mass spectrometer operated in electron impact mode with an electron energy of 70 eV and an emission current of 35 µA. The mass spectrometer scanned from *m/z* 29 to *m/z* 400 at 1.9 scans/s. Compounds were identified by comparing their mass spectrum and linear retention index (LRI) with those of authentic standards. Approximate quantities of the volatiles in the headspace were estimated by comparison of their peak areas with that of the 1,2-dichlorobenzene internal standard, obtained from the total ion chromatograms, using a response factor of 1.

Analysis of Free Amino Acids and Sugars

The concentrations of free amino acids, glucose and ribose in the raw chunks were determined by methods described by Elmore *et al.* (*16*).

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Results and Discussion

2-Furfural

There are a number of possible pathways for the formation of 2-furfural. The simplest is the dehydration of pentoses, which is described by a number of authors (*1*, *17*, *18*). This reaction proceeds via the intermediate 3-deoxypentosone and, after cyclisation and dehydration, the resulting molecule of 2-furfural contains the original carbon skeleton of the pentose in-tact. It is also possible that the 3-deoxypentosone can be formed by recombination of a C2 and a C3 sugar fragment which are formed from breakdown of either hexoses or pentoses. There are also some instances where 2-furfural has been formed from an in-tact hexose sugar either by thermal degradation of 5-hydroxymethyl-2-furfural (*19*) or by cleavage of the hexose Amadori rearrangement product between C1 and C2 of the original hexose (*20*). Depending on which of these pathways predominates, the formation of 2-furfural may be influenced by changes in the glucose concentration.

Figure 1a shows the effect of adding two levels of glucose to the raw material and also the effect of adding two levels of glycine at each of the glucose levels. At level 1 glucose (L1, 5 mmol/100 g), the concentration of 2-furfural doubled compared to the control (L0) and the added glycine had no significant impact. At level 2 glucose (L2, 20 mmol/100 g), the concentration was ten times greater than the control but there was a significant decrease with added glycine. This shows that in this food matrix, the concentration of 2-furfural can be increased by adding glucose.

This was confirmed by examining the mass spectrum of 2-furfural formed in a similar system containing $^{13}C_6$ glucose, dosed at a concentration equivalent to L1 (Figure 1b). Based on the amount of glucose added and the endogenous glucose in the system, 80% of the glucose molecules were fully labeled.

The spectrum of unlabeled 2-furfural contains two strong M^+ and $(M-1)^+$ ions in roughly equal proportions which, in the unlabeled molecule, are at m/z 95 and 96. In the 2-furfural isotopomer which comprises five labeled carbon atoms, these shift to 100 and 101. In the case where only some of the carbon atoms are labeled, there would be M^+ and $(M-1)^+$ ions at m/z 97 and 98 (two labels) and m/z 99 and 100 (three labels). Figure 1b shows that there were only two isotopomers present: the unlabeled isotopomer and the quintuply labeled isotopomer in a ratio of approximately 4:5. There was no evidence of any isotopomers which had been formed from the recombination of sugar fragments.

Whilst approximately 45% of the 2-furfural was unlabeled, only 20% of the original glucose was unlabeled, so the remainder of the unlabeled 2-furfural must have been derived from another source, most probably the low levels of ribose (0.004 mmol/100g) present in the raw materials. Thus, approximately 55% of the 2-furfural was formed from in-tact labeled glucose, 15% from the in-tact unlabeled glucose and 30% from ribose. In summary, in this system 2-furfural is formed from both hexoses and pentoses, but the fact that 30% is derived from 0.004 mmol/100g of ribose, whereas the remaining 70% requires addition of 5 mmol/100 g of glucose, demonstrates the higher efficiency of the ribose pathway compared with the glucose pathway. It should be noted that whereas 2-furfural is a major product in the ribose pathway, it is one of many products formed from glucose. Thus, ribose is a key precursor for the formation of 2-furfural in petfood, but addition of sufficient quantities of glucose can also lead to an increase in its formation.

Glycine has multiple roles in the Maillard reaction, one of the most important being its role as a catalyst in the early stages of the reaction. However, in this system, glycine did not alter the formation of 2-furfural, except when glucose was added at L2 in which case the high concentration of glycine decreased the amount of 2-furfural formed. An increase in the concentration of both glucose and glycine is likely to increase the formation of formaldehyde through Strecker degradation. This highly reactive intermediate can react with many of the intermediates formed during the formation of 2-furfural, thus reducing its rate of formation. Thus addition of glycine to enhance the early stages of the Maillard reaction can reduce the amount of 2-furfural formed in the final product.

Pyrazines

Pyrazines are responsible for many roasty, toasty and nutty notes in cooked foods and have attracted much attention. Weenen (*21*) used ^{13}C labeling in model systems to determine the major carbohydrate cleavage pathways involved in pyrazine formation. Chui et al. (*22*) elucidated the mechanism of alkylpyrazine formation in model systems and postulated both the oxidative and non-oxidative routes to pyrazine formation (Figure 1). This was later confirmed in labeling studies by others, including Amrani-Hemaimi (*7*) and Cerny (*23*). Parker *et al.* (*15*) were able to distinguish between pyrazines formed predominantly from the oxidative pathway and those formed predominantly from the non-oxidative pathway in meat systems. Similar results were found by Low *et al.* ((*24*) who studied pyrazine formation in potato cakes. This group used [2-^{13}C]glycine to show that the dominant mechanism for the formation of simple pyrazines tended to be the reaction between two amino ketones forming a dihydropyrazine. The dihydropyrazine was subsequently oxidized to form the pyrazine without the involvement of any other precursors. This was termed the oxidative route or “x+x” pathway, where x represents the two aminoketones (see Figure 2). More highly substituted pyrazines were also formed but, in this case, further substituents were incorporated into the molecule via condensation of the dihydropyrazine with another aldehyde such as formaldehyde or acetaldehyde without the need for

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oxidation. This was termed the non-oxidative route or “x+x+y” pathway where x represents the aminoketone and y the aldehyde, which can be lipid or Strecker derived. Thus some pyrazines may be sensitive to the addition of glucose whereas for others the limiting precursor may be the availability of the aldehyde.

Both oxidative and non-oxidative pathways were shown to be involved in the formation of pyrazines during the processing of petfood. Figure 3 shows the result of adding two levels of glucose to the raw material and also the effect of adding two levels of glycine to each of the glucose levels. The 2,5- and 2,6-dimethylpyrazines (Figure 3a) increased with L1 glucose but there was no further increase with L2. There was no significant difference when glycine was added at either L1 or L2. This is consistent with the findings of Low *et al.* (*24*). These simple disubstituted pyrazines were not influenced by additional glycine because they are predominantly formed via the oxidative route which involves only aminoketones (derived directly from glucose via the Strecker degradation). In contrast to this, the trimethylpyrazine (Figure 3b) was not sensitive to added glucose but increased linearly with added glycine. This demonstrates the requirement for formaldehyde (the Strecker degradation product of glycine) and suggests that the dominant mechanism for trimethylpyrazine is the non-oxidative “x+x+y” pathway.

These observations were confirmed by examining the mass spectra of the pyrazines formed in the system containing $^{13}C_6$ labeled glucose (Figure 4). Based on the fact that 80% of the glucose was fully labeled, it was assumed that 80% of the methylglyoxal was also fully labeled. If the only pathway for 2,5-dimethylpyrazine is the oxidative pathway, then it would be formed from two molecules of methylglyoxal. The major isotopomer would be formed from the condensation of two fully labeled units of methylglyoxal and would contain six labeled carbon atoms and an M^+ of 114. This would account for approximately 64% (80% x 0.80) of the 2,5-dimethylpyrazine formed. The unlabeled isotopomer (M^+ of 108) would account for 4% (20% x 0.20) and the remaining 32% would be formed from one labeled molecule of methylglyoxal and one unlabeled giving an M^+ of 111. This is very close to the ratio that was found (Figure 4a) but there was also approximately 7% of the isotopomer with M^+ of 113 present in the mass spectrum. This isotopomer is formed from the non-oxidative pathway by the condensation of one molecule of methylglyoxal, one molecule of glyoxal (both of which are 80% labeled) and one molecule of unlabeled formaldehyde. The result is the formation of isotopomers with M^+ of 113, 111, 110 and 108 in a ratio of 64:16:16:4 respectively, but the less abundant isotopomers were not observed in the mass spectrum. It can be estimated from the mass spectrum that about 90% of the 2,5-dimethylpyrazine was formed from the oxidative pathway and 10% from the non-oxidative pathway involving formaldehyde. This small contribution from the non-oxidative pathway explains why the formation of 2,5-dimethylpyrazine was not sensitive to the addition of glycine (Figure 3a).

The formation of trimethylpyrazine is more complex, but the same logic can be applied. The oxidative pathway involves the incorporation of one molecule of 2,3-butanedione and one of methylglyoxal. The non-oxidative pathway involves either two molecules of methylglyoxal and one of formaldehyde, or one molecule of 2,3-butanedione, one of glyoxal and one of formaldehyde giving three possible

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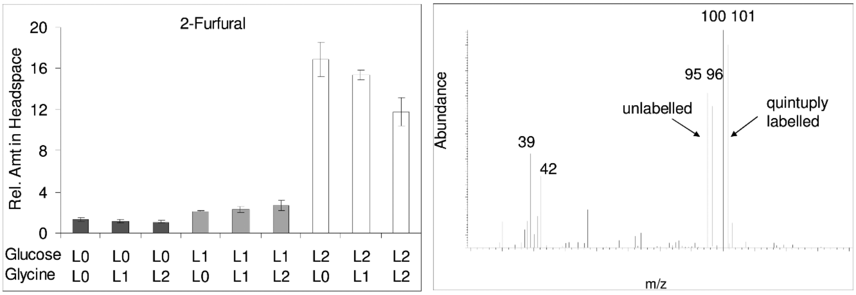

Figure 1. a) Relative amount of 2-furfural in the headspace of nine canned products with three levels of added glucose (L0, L1, L2) and three levels of added glycine (L0, L1, L2). b) Mass spectrum of 2-furfural when extract spiked with 80 % $^{13}C_6$ glucose

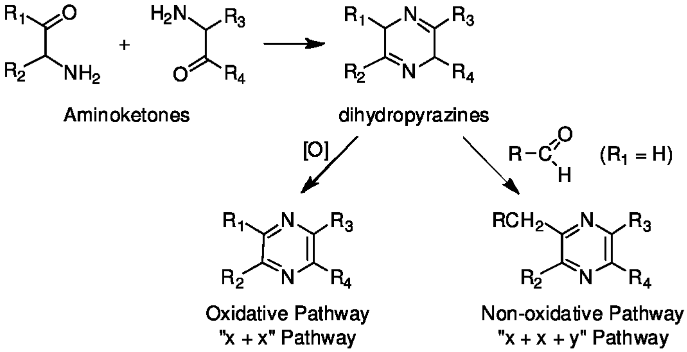

Figure 2. Two possible mechanisms for alkylpyrazine formation: "x + x" involving condensation of two aminoketones derived from dicarbonyls (x) to form a dihydropyrazine, followed by direct oxidation; and "x + x + y" involving incorporation of an additional substituent through interaction of an aldehyde (y) with the dihydropyrazine intermediate.

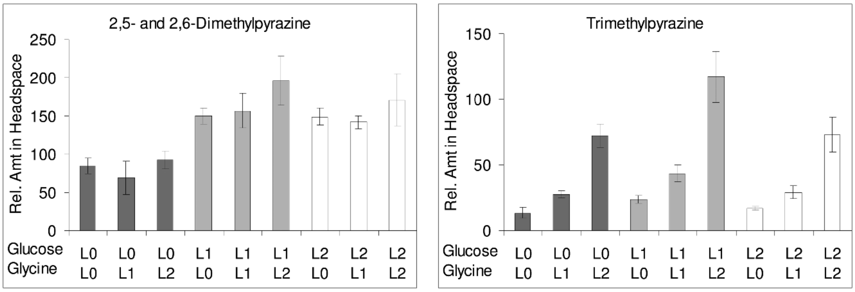

Figure 3. Relative amount of a) 2,5- and 2,6-dimethylpyrazine and b) trimethylpyrazine in the headspace of nine standard canned products with three levels of added glucose (L0, L1, L2) and three levels of added glycine (L0, L, L2)

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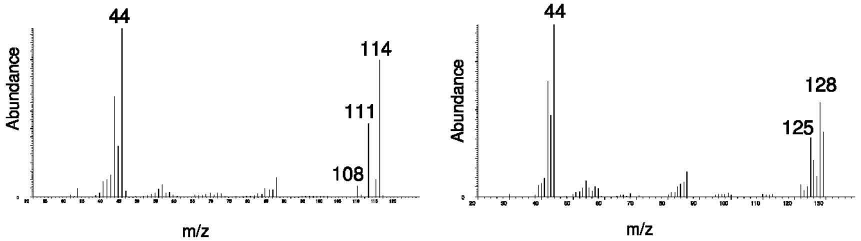

Figure 4. Mass spectrum of a) 2,5-dimethylpyrazine and b) trimethylpyrazine formed in the extract containing 80% $^{13}C_6$ glucose

combinations of precursors. The mass spectrum of 2,3-butanedione in the labelled extract showed it to be about 50% quadruply labeled (from β-cleavage of 1-deoxyglucosone) and 50% triply labeled (from the aldol condensation of methylglyoxal and formaldehyde). Using the same approach as was used with the 2,5-dimethylpyrazine, it can be estimated that about 60% of the trimethylpyrazine was formed from the oxidative pathway and about 40% from the non-oxidative pathway involving the incorporation of formaldehyde into the dihydropyrazine. However, in this case, the oxidative pathway also involved formaldehyde as it was incorporated into 50% of the 2,3-butanedione molecules prior to the formation of aminoketones. Thus, the formation of trimethylpyrazine is much more dependent on the availability of formaldehyde and this was indeed observed in the standard cans of petfood where the addition of glycine had a major impact on the amount formed (Figure 3b).

This study used a combination of small-scale ^{13}C labeling experiments and addition of key precursors on a larger scale to determine the dominant formation pathways and limiting precursors for 2-furfural, 2,5-dimethylpyrazine and trimethylpyrazine in a complex food matrix.

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Chapter 10

The Effects of Storage on the Formation of Aroma and Acrylamide in Heated Potato

J. Stephen Elmore,*,[1] Andrew T. Dodson,[1] Adrian Briddon,[2] Nigel G. Halford,[3] and Donald S. Mottram[1]

**[1]Department of Food and Nutritional Sciences, University of Reading,
Whiteknights, Reading RG6 6AP, United Kingdom
[2]Potato Council, Sutton Bridge Experimental Unit, East Bank,
Sutton Bridge, Spalding PE12 9YD, United Kingdom
[3]Crop Performance and Improvement Division, Rothamsted Research,
Harpenden AL5 2JQ, United Kingdom
*j.s.elmore@reading.ac.uk**

In fried and baked potatoes the Maillard reaction gives desirable aroma, while asparagine reacts with sugars to give acrylamide, a suspected carcinogen. Sugars and free amino acids were measured in unstored potatoes, and potatoes stored for 1 month at 4°C and 12°C. Reducing sugars in tubers stored at 4°C and 12°C increased by ten and two times, respectively. Changes in free amino acids were smaller, although asparagine increased at both temperatures. Formation of acrylamide and aroma compounds in heated potato flour was dependent on levels of total sugars, relative to levels of total free amino acids. When sugar levels were relatively high, i.e., in samples stored at 4°C, acrylamide and aroma compound formation were proportional to sugar concentration. When sugar levels were low, acrylamide and aroma compound formation were proportional to the concentrations of their precursor amino acids, expressed as a percentage of total free amino acids.

Introduction

The presence of the potential carcinogen acrylamide in baked and fried potatoes is still a source of concern for the food industry. Research worldwide

In Controlling Maillard Pathways To Generate Flavors; Mottram, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

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continues to find ways to reduce its formation during the cooking process (*1*, *2*) and to develop new cultivars, which are low in its precursors, asparagine and reducing sugars (*3*).

Levels of acrylamide in cooked potatoes have been shown by many authors to be related to the amount of reducing sugars in the uncooked potato (*4–6*). Furthermore, it is widely recognized that reducing sugar levels increase in potatoes stored at refrigerator temperatures, a phenomenon known as cold sweetening (*7*, *8*). However, storage at such low temperatures is often necessary, in order to prevent tuber sprouting and/or deterioration. Although sprouting suppressants, such as chlorpropham (CIPC) are necessary for long-term storage of tubers, their use needs to be carefully managed, as issues exist regarding possible toxicity and environmental contamination (*9*). Hence, storage regimes that restrict increases in reducing sugars, yet prevent sprouting, are sought.

Acrylamide is formed during the Maillard reaction, as are many important aroma and color compounds. It is rarely acknowledged that acrylamide mitigation strategies may involve excessive control of the Maillard reaction, resulting in cooked products with undesirable sensory attributes.

This paper examines how storage for 1 month, at both 12°C and 4°C, affects the sugar and free amino acid composition of Santé potatoes, and the resulting effects on acrylamide levels and Maillard-derived aroma compounds in cooked potato.

Materials and Methods

Potato Samples

Potatoes (cultivar Santé) were hand-harvested from a site near Spalding, Lincolnshire, in October, 2005, from four plants. The tubers from each plant were divided into 3 groups, so that the average size of the tubers in each group was similar. The first group (T_4) was stored for 1 month at 4°C, the second (T_{12}) was stored for 1 month at 12°C and the third (*C*) was not stored but was prepared for freeze drying within 48 hours of lifting. Storage took place in plastic trays at the experimental stores of Sutton Bridge Experimental Unit, Spalding. Stores operated under discontinuous refrigeration/ventilation, i.e., fridge and fan operated when store temperature exceeded 0.2°C above the set point.

Preparation of Samples for Analysis

Potato samples from each plant and storage regime were separately freeze-dried prior to analysis, resulting in twelve different samples. Potatoes were peeled, sliced and placed on aluminum trays, frozen in a blast freezer for 15 min and freeze-dried for 3 days. The dried samples were chopped using a food processor, vacuum sealed separately in pouches and stored at –18°C until analysis.

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Determination of Free Amino Acids

Freeze-dried samples (0.500 ± 0.005 g) were weighed into a 14-mL screw-top bottle. Hydrochloric acid (10 mL, 0.01 M) was added to the vial and the sample was stirred for 15 min at room temperature. An aliquot of supernatant (2 mL) was centrifuged at 7200 *g* for 15 min and 100 µL of the centrifuged supernatant were then derivatized using the EZ-Faast amino acid derivatization technique (Phenomenex, Torrance, CA). Gas chromatography-mass spectrometry (GC-MS) analysis of the derivatized samples was carried out using an Agilent 5975 system in electron impact mode (Agilent, Santa Clara, CA). An aliquot of the derivatized amino acid solution (1 µL) was injected at 280°C in split mode (40:1) onto a Zebron ZB-AAA capillary column (10 m × 0.25 mm; 0.25 µm film thickness). The oven temperature was held at 110 °C for 1 min and then increased at 30°C/min to 310°C. The transfer line and ion source were maintained at 320°C and 230°C, respectively. Carrier gas flow rate was kept constant throughout the run at 1.5 mL/min. Three analyses were performed for each sample. This technique is not suitable for the determination of free arginine.

Analysis of Sugars by Ion Chromatography

Each flour sample (0.200 ± 0.005 g) was weighed into a 14-mL screw-top bottle. Aqueous methanol (50%) containing 100 mg/L trehalose was added to the bottle and the sample was stirred for 15 min at room temperature. After a further 15 min, 1.5 mL of supernatant was removed from the bottle and centrifuged at 7200 g for 15 min. Five hundred microliters of the centrifuged supernatant were diluted ten-fold in water; 2 mL of the diluted extract were then filtered through a 0.45 µm syringe filter.

The extracts were analyzed using a Dionex ion chromatography system with a 250 × 4 mm Carbopac™ PA1 column (Dionex Corporation, Sunnyvale, CA), operated using Chromeleon™ software. The ion chromatography system consisted of an AS50 autosampler, LC25 column oven, GS50 pumps, and an ED50 pulsed amperometric detector, running in internal amperometric mode. Injection volume was 25 µL. A gradient program was set up using 200 mM NaOH (solvent **A**) and water at a flow rate of 1 mL/min; 50 % solvent **A**, held for 10 min and then increased to 100 % at 40 min. The column was then washed for 8 min with 500 mM sodium acetate in 125 mM NaOH and re-equilibrated with 50 % solvent A for 7 min. The waveform of the pulsed amperometric detector was: 400 ms at 0.1 V, 20 ms at –2.0 V, 10 ms at 0.6 V and 60 ms at –0.15 V. Standards of glucose, fructose and sucrose were used for quantification. One analysis was performed for each sample.

Preparation of Cooked Potato Samples for Flavor and Acrylamide Analysis

Potato flour samples (0.5 g) in unsealed glass ampoules (1 mL capacity) were heated for 20 min, at 160°C. Copper wire was tied around the necks of the filled ampoules, and the ampoules were then heated by being suspended by the wire from the ceiling of a GC oven. For acrylamide analysis three replicates were prepared for each of the twelve flour samples; while only one volatile analysis was performed for each sample.

Analysis of Acrylamide

Acrylamide was extracted from these samples with 50% aqueous methanol and converted to the dibromo derivative, prior to analysis by GC-MS. Labeled $^{13}C_3$-acrylamide was used as the internal standard. Acrylamide was extracted from these samples with 25 % aqueous methanol and converted to the dibromo-derivative, prior to analysis by GC-MS, using the method of Castle *et al.* (*10*), with the modifications described by Elmore *et al.* (*11*). Labeled $^{13}C_3$-acrylamide was used as the internal standard.

The brominated extracts (2 µL) were injected onto the Agilent 5975 GC-MS system in pulsed splitless mode at 250°C, the splitter opening after 0.5 min. The helium carrier gas pressure was 21 psi in pulsed mode, falling to 9.6 psi for the rest of the run. A DB-17 MS capillary column was used (30 m × 0.25 mm i.d., 0.15 µm film thickness; Agilent). The oven temperature was 85 °C for 1 min, rising at 8 °C/min to 200°C, then 30 °C/min to 280°C for 10 min. The transfer line was held at 280°C and the ion source at 180°C. The mass spectrometer was operated in electron impact mode with selected ion monitoring. Two ions were used to monitor brominated $^{13}C_3$-acrylamide (*m/z* 153 and 155) and another two ions were used for brominated acrylamide (*m/z* 150, 152). The ion *m/z* 155 was used to quantify brominated $^{13}C_3$-acrylamide and the ion *m/z* 150 was used to quantify brominated acrylamide.

Analysis of Volatiles

Aroma isolates were collected on Tenax TA. The cooked flour was transferred to a 250-mL conical flask with a screw-thread neck and 100 mL of HPLC-grade water were added. Nitrogen, at 40 mL min^{-1}, flowed over the cooked flour sample, which was held at 35°C, for 45 min, sweeping volatiles onto a glass trap (3.5” × 0.25” i.d.) containing 85 mg Tenax TA (Supelco, Bellefonte, PA). A standard (100 ng 1,2-dichlorobenzene in 1 µL methanol) was added to the trap at the end of the collection, and excess solvent and any water retained on the trap were removed by purging the trap with nitrogen at 100 mL min^{-1} for 10 min.

All analyses were performed on a PerkinElmer Clarus 500 GC-MS system (PerkinElmer Life and Analytical Sciences Inc., Waltham, MA) running Turbomass software (Version 4.5; PerkinElmer). A PerkinElmer Turbomatrix automated thermal desorber (ATD) was used to transfer the volatiles from the Tenax trap onto the front of a J & W DB-5 fused silica capillary column (60 m × 0.32 mm i.d., 1 µm film thickness; Agilent Technologies, Santa Clara, CA), *via* a cold trap. In the ATD the Tenax trap was heated at 300°C for 10 min.; volatiles were desorbed onto the cold trap, which was held at -30°C. After desorption, the cold trap was then heated to 300°C at 40°C per second, to release the volatile material onto the GC column. After desorption, the temperature of the oven was held at 40°C for a further 2 min, before being raised at 4°C min^{-1} to 250°C. Helium at 21 psi was used as the carrier gas. A series of *n*-alkanes (C_5-C_{25}) in

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diethyl ether was analyzed, under the same conditions, to obtain linear retention index (LRI) values for the cooked potato aroma components.

The mass spectrometer operated in electron impact mode with an electron energy of 70 eV. The mass spectrometer scanned from *m/z* 29 to *m/z* 350 at 2.5 scans/s. Compounds were identified by first comparing their mass spectra with those contained in the NIST/EPA/NIH Mass Spectral Database or in previously published literature. Where possible, identities were confirmed by comparison of mass spectra and linear retention index (LRI) values with those of authentic standards, either purchased or synthesized. Approximate quantities of the volatiles in the headspace were estimated by comparison of their peak areas with that of the 1,2-dichlorobenzene internal standard, obtained from the total ion chromatograms, using a response factor of 1.

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Statistical Analysis

XLStat 2006 (Addinsoft, Paris, France) was used to perform analysis of variance on the data.

Results and Discussion

Amino Acids

Table I shows how free amino acids in potato flour were affected by storage. Free asparagine increased significantly during both storage regimes, by up to 50% at 4°C, although storage temperature had no significant effect on asparagine concentration.

Glutamine decreased sharply on storage, so that the combined molar concentration of these two compounds was unchanged. Although many of the amino acids were significantly affected by storage, changes in general were small. Glutamic acid showed the largest increase, doubling in concentration at both storage temperatures. Those amino acids that are regarded as important participants in flavor formation, i.e., glycine, alanine, valine, leucine, isoleucine, phenylalanine, methionine, serine and threonine, tended to decrease at 12°C and increase at 4°C. This effect was not significant for glycine and valine, and was not observed for threonine and phenylalanine, the former decreasing on storage and the latter increasing. Some plant-to-plant variation was observed, but this was relatively small; several free amino acids were at lower concentrations in one particular plant, compared with the rest, but only in the control and at 12°C. No plant effect was observed for asparagine (data not shown).

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Table I. Free amino acids (mmol per kg dry weight) in potato (var. Santé)

Amino acid	*Treatment*[a]			*Effect of storage*[b]
	C	T_4	T_{12}	
Asparagine	100 (6.64)[c]	148 (19.2)[d]	135 (17.3)[d]	***
Glutamine	80.5 (9.36)[e]	31.6 (15.5)[c]	44.1 (9.73)[d]	***
Alanine	2.63 (0.91)[c,d]	3.29 (0.44)[d]	2.52 (0.65)[c]	*
Glycine	1.04 (0.21)	1.08 (0.31)	0.91 (0.19)	NS
Valine	9.57 (1.57)	11.1 (1.28)	10.9 (2.69)	NS
β-Alanine	0.28 (0.13)	0.23 (0.17)	0.24 (0.19)	NS
Leucine	1.61 (0.29)[d]	2.11 (0.30)[e]	1.17 (0.18)[c]	***
Isoleucine	2.99 (0.45)[c]	3.62 (0.53)[d]	2.82 (0.66)[c]	**
Threonine	3.93 (0.32)[d]	2.40 (0.48)[c]	2.37 (0.51)[c]	***
Serine	2.94 (0.68)[d]	4.35 (1.25)[e]	2.01 (0.54)[c]	***
GABA	10.3 (2.20)[d]	9.39 (1.21)[d]	7.01 (1.41)[c]	***
Proline	2.15 (0.51)[c]	3.64 (0.52)[d]	3.23 (0.78)[d]	***
Aspartic acid	16.0 (0.87)[d]	15.8 (2.29)[d]	10.8 (1.23)[c]	***
Methionine	3.04 (0.39)[c,d]	3.56 (0.60)[d]	2.92 (0.68)[c]	*
Glutamic acid	17.8 (1.94)[c]	32.6 (4.85)[d]	34.5 (5.79)[d]	***
Phenylalanine	3.18 (0.51)[c]	5.84 (0.85)[e]	4.76 (1.17)[d]	***
Ornithine	0.41 (0.10)[d]	0.19 (0.08)[c]	0.12 (0.03)[c]	***
Lysine	4.23 (0.80)[d]	1.57 (0.38)[c]	1.38 (0.32)[c]	***
Histidine	1.04 (0.31)[d]	0.53 (0.20)[c]	0.50 (0.15)[c]	***
Tyrosine	2.08 (0.24)	2.18 (0.83)	1.73 (0.52)	NS
Tryptophan	1.65 (0.24)[d]	0.74 (0.33)[c]	0.74 (0.25)[c]	***
TOTAL	268 (18.4)	284 (42.7)	270 (39.6)	NS

[a] Mean values are shown, with standard deviations in parentheses. [b] Probability that there is an effect of storage; NS, no difference between means ($p > 0.05$); *, significant at the 5% level; **, significant at the 1% level; ***, significant at the 0.1% level. [c] Means ($n = 12$) in the same row labeled with different superscripts are different ($p < 0.05$). [d] Means ($n = 12$) in the same row labeled with different superscripts are different ($p < 0.05$). [e] Means ($n = 12$) in the same row labeled with different superscripts are different ($p < 0.05$).

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Table II. Sugars (mmol per kg dry weight) in potato (var. Santé)[a]

Sugar	*Treatment*		
	C	*T_4*	*T_{12}*
glucose	7.59 (4.75)	84.1 (8.73)	13.4 (4.02)
fructose	3.15 (1.93)	64.5 (5.26)	7.82 (1.62)
sucrose	20.5 (1.67)	27.7 (0.80)	18.0 (1.27)
TOTAL	34.8 (6.86)	181 (14.4)	44.8 (6.91)

[a] Mean values of four analyses are shown, with standard deviations in parentheses.

Table III. Acrylamide (mg/kg) in potato flour (var. Santé) cooked for 20 min at 160 °C

Treatment	*Acrylamide[a]*	
	dry weight	*wet weight[b]*
C	5.03 (1.32)	1.06 (0.277)
T_4	30.0 (1.52)	6.57 (0.333)
T_{12}	6.75 (0.808)	1.44 (0.172)

[a] Mean values of twelve analyses (3 replicates from 4 plants) are shown. [b] Concentration calculated from moisture losses due to freeze drying.

Sugars

Both reducing sugars exhibited a large increase at 4°C, particularly fructose (Table II). A smaller, but still highly significant increase in sucrose was also observed at 4°C. The large effect of storage at 4°C skewed the data, preventing a proper comparison of the unstored and the 12°C samples. When these samples were compared alone, a significant increase in fructose was also observed at 12°C, but the other sugars did not alter. Plant-to-plant variation was not measured for sugars.

Acrylamide

Table III shows the mean values for acrylamide across the four plants for each storage treatment. Storage at 12°C and 4°C led to an increase in acrylamide when the samples were heated, compared with the unstored sample. Storage at 12°C led to an increase of 30% in the amount of acrylamide produced, whereas the level of acrylamide in the potatoes stored at 4°C was nearly six times higher than in the control.

Two-way analysis of variance, examining storage and between-plant variation, showed that both effects and their interaction were highly significant (data not shown). One plant gave the highest levels of acrylamide on cooking at all temperatures. This plant also contained the highest concentration of reducing sugars and total sugars in all three treatments.

As the samples were freeze-dried and then cooked, moisture levels were very low and acrylamide formed rapidly, giving values that would be far higher than those measured in, for example, cooked french fries. Moisture losses during freeze drying were measured, allowing wet weight values to be calculated (moisture content of the tubers was 78.6±1.3%).

Aroma Compounds

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Forty-three compounds assumed to be formed *via* the Maillard reaction are presented in Table IV. All of these compounds were present in at least one of the headspace extracts of the heated potato flour with an average total ion chromatogram peak area greater than or equal to 5% of that of 100 ng of internal standard. Thirty-one compounds were affected by storage; we have reported many of these compounds previously in heated potato flour and heated whole wheat flour (*12*, *13*) and demonstrated how they may be formed from amino acid decomposition. For example, valine can decompose to 2-methylpropanal, which oxidizes to give 2-methylpropanoic acid, condenses with alanine to give 4-methyl-2-pentenal, and with methionine to give 2-methyl-2-(methylthio)propanal. 2-Methylpropanal also forms the isobutyl group in 1-isobutylpyrrole, 1-isobutyl-3-methylpyrrole, 2-methyl-3-isobutylpyrazine, and 2,5(*or* 6)-dimethyl-3-isobutylpyrazine. The compound tentatively identified as 1-isobutyl-3-methylpyrrole has not been reported in heated potato before but is present in roasted coffee (*14*).

All of the compounds affected by storage were at highest concentrations in the T_4 samples. Generally, compounds were lowest in T_{12} samples but not significantly so. However, 3-methylbutanal and (*E*)-4-methyl-2-hexenal were significantly reduced in the T_{12} samples. In addition, heated T_{12} flour was slightly darker in color than *C* flour, although heated T_4 flour was much darker than the other two flours. Between-plant variation was not measured for aroma compounds.

Table IV. Effect of storage on the volatile aroma compounds in the headspace of potato flour (var. Santé) cooked for 20 min at 160 °C

		Mean concentration in headspace[a]				
LRI[b]	*Compound*[c]	*C*	T_4	T_{12}	*SE*[d]	*P*[e]
<500	*Sulfur dioxide*	22.9	13.9	19.0	2.7	NS
500	Acetone	10.3	13.0	6.9	1.4	NS
554	2-Methylpropanal	436[f]	835[g]	396[f]	76.3	*
581	Acetic acid	9.0	8.1	5.1	1.0	NS

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Table IV. (Continued). Effect of storage on the volatile aroma compounds in the headspace of potato flour (var. Santé) cooked for 20 min at 160 °C

		Mean concentration in headspace[a]				
LRI[b]	*Compound*[c]	*C*	T_4	T_{12}	*SE*[d]	*P*[e]
596	2-Butanone	11.3^{f}	22.4^{g}	7.9^{f}	2.2	**
603	2-Methylfuran	4.4^{f}	20.9^{g}	2.9^{f}	2.7	***
611	3-Methylfuran	1.2^{f}	8.4^{g}	1.0^{f}	1.1	***
654	3-Methylbutanal	399^{g}	611^{h}	287^{f}	42.3	***
665	2-Methylbutanal	794^{f}	1710^{g}	701^{f}	143	***
696	2,3-Pentanedione	0.7^{f}	5.5^{g}	0.4^{f}	0.7	***
723	2-Vinylfuran	$4.0^{f,g}$	8.9^{g}	1.5^{f}	1.3	*
738	1-Methylpyrrole	5.4	3.5	2.8	0.8	NS
743	2-Methylpropanoic acid	22.5	22.1	5.1	3.6	NS
746	Dimethyl disulfide	151^{f}	386^{g}	121^{f}	39.5	***
748	Pyrrole	7.7	6.1	4.0	0.8	NS
769	Toluene	20.9	16.2	21.1	1.3	NS
816	*(E)-4-Methyl-2-pentenal*	3.1^{f}	7.2^{g}	2.3^{f}	0.7	***
827	3-Methylbutanoic acid	7.4	6.7	2.4	1.0	NS
828	Methylpyrazine	4.8^{f}	17.4^{g}	3.7^{f}	1.9	***
840	2-Methylbutanoic acid	18.4	21.4	5.7	3.2	NS
856	2-Methyl-2-(methylthio)propanal	$3.8^{f,g}$	5.2^{g}	2.3^{f}	0.4	**
875	2-Isopropyl-(*E*)-2-butenal	5.4^{f}	15.9^{g}	3.6^{f}	1.7	***
898	Styrene	4.9	5.8	5.4	0.7	NS
917	2,6(*and* 2,5)-Dimethylpyrazine	8.1^{f}	26.4^{g}	6.6^{f}	2.8	***
918	(*E*)-4-Methyl-2-hexenal	3.2^{g}	7.5^{h}	1.9^{f}	0.7	***
919	(*E*)-5-Methyl-2-hexenal	2.9^{f}	6.4^{g}	2.1^{f}	0.6	***
921	Ethylpyrazine	3.7^{f}	11.1^{g}	2.2^{f}	1.2	***
951	1-Isobutylpyrrole	$5.0^{f,g}$	11.0^{g}	2.3^{f}	1.4	*
972	Benzaldehyde	19.2	12.2	16.6	1.3	NS
982	Dimethyltrisulfide	$28.7^{f,g}$	47.6^{g}	16.9^{f}	4.6	**
1002	2-Ethyl-6-methylpyrazine	3.8^{f}	14.4^{g}	2.9^{f}	1.6	***
1006	2-Ethyl-3-methylpyrazine *and* trimethylpyrazine	3.7^{f}	11.3^{g}	2.8^{f}	1.2	***
1008	2-Ethyl-5-methylpyrazine	6.1^{f}	13.0^{g}	4.8^{f}	1.1	***

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Table IV. (Continued). Effect of storage on the volatile aroma compounds in the headspace of potato flour (var. Santé) cooked for 20 min at 160 °C

		Mean concentration in headspace[a]				
LRI[b]	*Compound*[c]	*C*	T_4	T_{12}	*SE*[d]	*P*[e]
1040	*1-Isobutyl-3-methylpyrrole*	1.1[f]	5.1[g]	0.6[f]	0.7	***
1053	Benzeneacetaldehyde	9.4	8.7	8.0	0.3	NS
1080	3-Ethyl-2,5(*or* 6)-dimethylpyrazine	6.7[f]	23.0[g]	6.2[f]	2.4	***
1083	2,6-Diethylpyrazine	1.9[f]	6.0[g]	1.1[f]	0.7	***
1141	2-Methyl-3-isobutylpyrazine	2.2[f]	7.5[g]	1.5[f]	0.8	***
1159	3,5 (*or* 6)-Diethyl-2-methylpyrazine	3.1[f]	10.2[g]	2.8[f]	1.0	***
1203	2,5(*or* 6)-Dimethyl-3-isobutylpyrazine	2.9[f]	7.5[g]	2.5[f]	0.7	***
1238	*3-Phenylfuran*	1.6[f]	5.7[g]	1.1[f]	0.6	***
1257	2-(3-Methylbutyl)-3-methylpyrazine	1.4[f]	4.9[g]	0.8[f]	0.6	***
1318	2,5(*or* 6)-Dimethyl-3-(3-methylbutyl)pyrazine	2.3[f]	5.6[g]	1.6[f]	0.6	***

[a] Values are the means of peak areas ($n = 4$) relative to that of 100 ng of 1,2-dichlorobenzene (peak area = 100) injected onto the Tenax trap after extraction. [b] Linear retention index on a J & W DB-5 fused silica capillary column (60 m × 0.32 mm i.d., 1 µm film thickness). [c] Compounds in italics are tentatively identified, based on mass spectrum (NIST05 database) and linear retention index. [d] Standard error of the mean ($n = 12$). [e] Probability that there is a difference between means; NS, no significant difference between means ($P > 0.05$); *, significant at the 5% level; **, significant at the 1% level; ***, significant at the 0.1% level. [f] Means in the same row with labeled with different superscripts are significantly different ($p < 0.05$). [g] Means in the same row labeled with different superscripts are significantly different ($p < 0.05$). [h] Means in the same row labeled with different superscripts are significantly different ($p < 0.05$).

Relationship between Acrylamide and Its Precursors

The relationship between reducing sugar levels and acrylamide formation in potatoes has been widely reported. However, when reducing sugars are at low concentrations relative to free amino acids, acrylamide formation has been shown to be proportional to the concentration of free asparagine, relative to the concentration of total free amino acids (*15*). When reducing sugar concentrations are higher than total amino acid concentrations, e.g., in potatoes that have undergone extended storage, the relationship between reducing sugars and acrylamide is also lost (*5*).

When the molar concentrations of total reducing sugars were plotted against acrylamide formed at 160 °C, a straight line was observed (Figure 1), indicating that reducing sugars were limiting under all treatment conditions. However,

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according to the extrapolated curve, acrylamide was still formed at relatively high levels, even when reducing sugar concentration was zero. When total sugars were plotted against acrylamide formed at 160 °C, a straight line was also observed (Figure 2). This time the trend line passed through the origin, suggesting that sucrose also contributed to the acrylamide yield on heating. The participation of sucrose in color formation in potato chips, *via* the Maillard reaction, has been previously reported (*16*). Hence, its participation in the formation of acrylamide is not unexpected.

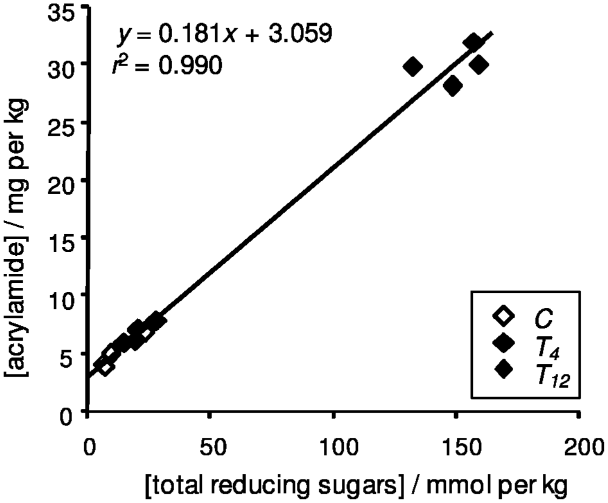

Figure 1. Correlation between total reducing sugars in uncooked potato and acrylamide in heated potato flour.

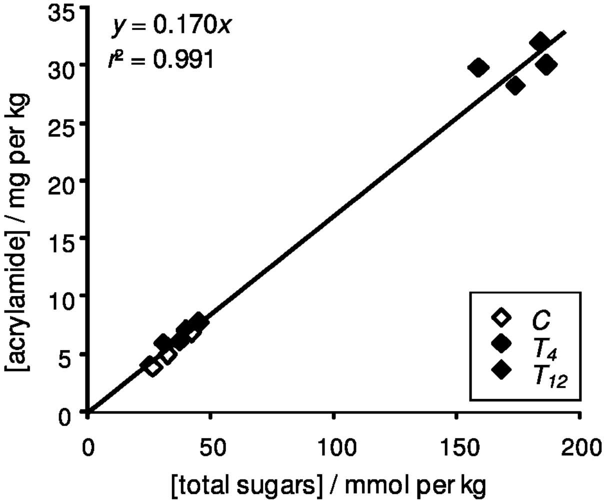

Figure 2. Correlation between total sugars in uncooked potato and acrylamide in heated potato flour.

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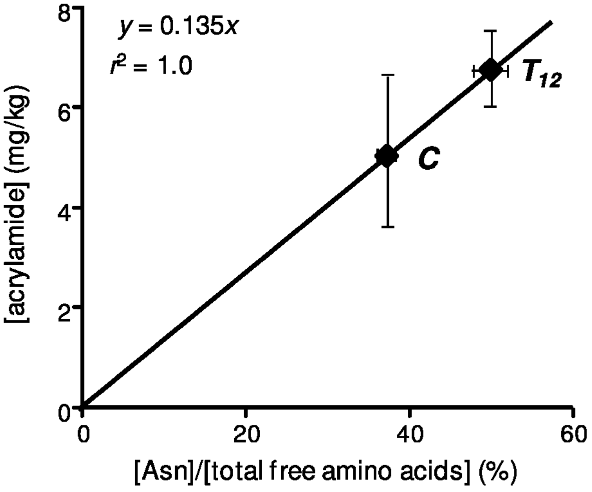

Figure 3. Correlation between relative free asparagine concentration in uncooked potato and acrylamide in heated potato flour.

Table V. Relative molar concentrations of sugars to free amino acids (FAA) in unstored and stored potato flour samples

Treatment	*[total reducing sugars]* *[total FAA]*	*[total sugars]* *[total FAA]*
C	0.040	0.15
T_{12}	0.079	0.12
T_4	0.52	0.62

The concentration of sugars and the amount of acrylamide formed from them was much higher in T_4 than in the other two samples. Hence, the effect of T_4 may have obscured what was happening in T_{12} and *C* samples when they were heated. Table V shows that total sugar concentrations, particularly total reducing sugars, were low relative to total free amino acid concentrations in *C* and T_{12}. For these two treatments the mean concentration of asparagine relative to the concentration of total free amino acids was considered and plotted against mean acrylamide formation (Figure 3). Although a lack of data meant that a two-point curve was plotted, the trend line passes through the origin with an r^2 value of 1, strongly suggesting that the molar concentration of asparagine relative to that of total free amino acids is a determinant of acrylamide formation in heated potatoes low in sugar.

This result indicates that substantial acrylamide formation will occur even when sugar concentrations are low but will be reduced in potatoes that are low in asparagine.

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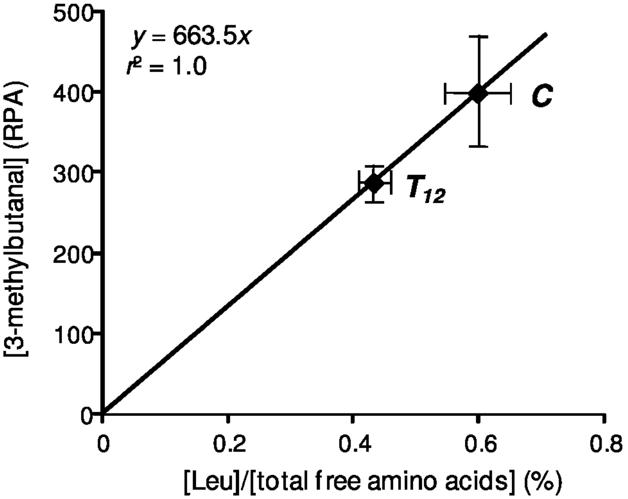

Figure 4. Correlation between relative free leucine concentration in uncooked potato and 3-methylbutanal in heated potato flour.

Relationship between Maillard-Derived Aroma Compounds and Their Precursors

When total sugars were plotted against the major Maillard-derived compounds formed at 160°C, a trend line passing through the origin, with an r^2 value greater than 0.90, was obtained for several compounds, in particular alkylpyrazines and compounds derived from sugars, such as furfural, 2-methylfuran and 2,3-pentanedione. Again, the participation of sucrose in the Maillard reaction appeared to be taking place, because when total reducing sugars rather than total sugars were plotted against aroma compounds formed, the correlation coefficients decreased in value. For other compounds, such as Strecker aldehydes, aldol condensation products (e.g., 5-methyl-2-hexenal and 2-isopropyl-2-butenal) and dimethyl disulfide and dimethyl trisulfide, a high linear correlation was observed when the trend line was not forced through the origin, indicating that their formation could occur even when sugars were not present. Some compound groups showed inconsistent effects. Pyrrole and 1-methylpyrrole showed no effect of sugar content, while 1-isobutyl-3-methylpyrrole was highly correlated with sugar content. Likewise, phenylacetaldehyde, the Strecker aldehyde of phenylalanine, showed no effect of sugar, whereas 3-phenylfuran, a condensation product of phenylalanine and a sugar fragment showed a high correlation with sugar content ($y = 0.0316x$; $r^2 = 0.972$).

The amounts of aroma compound formed at T_4 were up to eight times higher than in the other two samples. Again we considered *C* and T_{12} separately from T_4. Although heated T_{12} was higher in sugars than *C*, browner than *C* and contained more acrylamide than *C*, most Maillard-derived aroma compounds were lower in T_{12} than *C*, although only significantly in the case of 3-methylbutanal and (*E*)-4-

methyl-2-hexenal. Hence, once T_4 data was removed from the plot of sugar content *versus* aroma compound formation, all correlations were lost.

3-Methylbutanal has been widely reported as the Strecker aldehyde of leucine, while (*E*)-4-methyl-2-hexenal is an aldol condensation product of 2-methylbutanal and acetaldehyde from isoleucine and alanine, respectively (*13*). For *C* and T_{12} the mean concentration of leucine, relative to the concentration of total free amino acids, was plotted against mean 3-methylbutanal formation (Figure 4). Again a two-point curve was plotted; the trend line passed through the origin with an r^2 value of 1, suggesting that the molar concentration of leucine relative to that of total free amino acids is a determinant of 3-methylbutanal formation in heated potatoes low in sugar. (*E*)-4-Methyl-2-hexenal is formed from the condensation of two Strecker aldehydes and it was not possible to relate its formation to the relative concentration of either of its precursor amino acids.

Although not conclusive, these results again suggest that, at low sugar concentrations, competition between free amino acids for sugar fragments influences Maillard-derived compound formation. As within-sample variability was relatively high for sample *C*, definite conclusions are difficult to make.

Conclusions

Because only three sample sets were examined in this work, the following conclusions, regarding the roles of precursors in the formation of Maillard reaction products, are made with caution, until additional work, examining other storage temperatures and times, has been carried out.

Sugars increase substantially in potatoes during storage, particular under refrigerated conditions, while changes in free amino acids are relatively small. As a result, both the formation of Maillard-derived aroma compounds and acrylamide in cooked potatoes proceed in the same three ways, depending on how much sugar is in the tuber:

1). At the start of the storage period, sugar concentrations in tubers are low and free amino acids will compete for sugar and sugar fragments when the potato is heated. The concentrations of acrylamide or flavor compound formed during the Maillard reaction depend on the molar concentrations of their precursor amino acids relative to the total molar concentration of free amino acids. For example, 3-methylbutanal formation is dependent on the relative amount of leucine present.

2). As sugar concentrations increase, the relative concentrations of free amino acids become less important; acrylamide and Maillard-derived aroma compound formation becomes proportional to total sugar concentration.

3). After long-term storage, total sugar concentrations increase above total free amino acid concentrations and, as a result, the concentrations of acrylamide and aroma compounds derived from free amino acids will remain constant. However, the concentrations of sugar-derived volatiles, such as furfural, diacetyl and 2-acetylfuran, will continue to increase.

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Hence, in order to reduce acrylamide in french fries and chips, varieties should be chosen that are low in free asparagine, relative to total free amino acids, and which are less susceptible to cold sweetening. Such strategies should result in potato products containing low acrylamide with no negative effects on aroma.

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Chapter 11

Methionine-Containing Cyclic Dipeptides: Occurrence in Natural Products, Synthesis, and Sensory Evaluation

Neil C. Da Costa,* Michael Z. Chen, Debra Merritt, and Laurence Trinnaman

International Flavors & Fragrances, Inc., 1515 Highway 36, Union Beach, NJ 07735, USA
***neil.dacosta@iff.com**

Cyclic dipeptides (2,5-diketopiperazines) have been documented as occurring in an ever increasing variety of foods and natural products, such as beef, beer, cocoa, coffee, and chicken. They are a group of thermally generated compounds whose occurrence appears to be linked to Maillard reaction generated compounds. Their contribution to flavor has been reported as weak taste effects, such as bitterness and astringency. However, their presence in many cooked foods makes them difficult to ignore. Of the few sulfur containing amino acids, only one methionine-containing cyclic dipeptide has been reported previously in nature. This was cyclo-(methionine-proline) in beer and dry aged beef. This work presents the synthesis of several other cyclo-methionine analogues, their sensory attributes and their occurrence in food products, such as beef, beer and autolyzed yeast.

Introduction

Cyclic dipeptides, also called 2,5-diketopiperazines, have been found in various foods. (*1–10*). They have been described primarily as bitter, with some astringent and drying notes. In 1989 Rizzi disclosed experimental work on their formation in foods (*11*). Their formation was described as thermally dependant, under acid conditions (Figure 1), and several experiments were conducted to prove this. It was shown that heating equimolar equivalents of amino acids

In Controlling Maillard Pathways To Generate Flavors; Mottram, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

did not produce cyclic dipeptides. However heating acylic dipeptides under acid conditions did produce the corresponding cyclic dipeptides. Also heating a tripeptide such as Ala-Leu-Gly led to the formation of cyclo-(Ala-Leu).

In 1997, Peppard *et al* found seven proline-based diketopiperazines in beer including cyclo-(Met-Pro) (*3*). They evaluated their sensory properties at concentrations from 10 to 50 ppm, and the compounds were described as having flavor bitter, mouth coating, drying and astringent characteristics. Ginz *et al.* reported the presence of ten diketopiperazines in roasted coffee although no sensory evaluation was reported (*7*, *8*). Chen *et al.* (2004) tentatively identified twenty diketopiperazines in chicken essence (*9*). Some of the diketopiperazines were quantified before and after thermal treatment of chicken essence, and it was found that the concentrations of the diketopiperazines were determined by the relative ease of formation and type of process used. Stark *et al.* provided detailed information on twenty-five diketopiperazines in roasted cocoa nibs (*5*, *6*). In addition, using taste recognition thresholds of the compounds, the data revealed that among the twenty-five diketopiperazines, five compounds, namely, *cis*-cyclo(L-Ala-L-Ile), *cis*-cyclo(L-Ala-L-Leu), *cis*-cyclo(L-Ile-L-Pro), *cis*-cyclo(L-Leu-L-Val) and cis-cyclo(L-Pro-L-Val), were present above their individual bitter taste threshold concentrations and, therefore, contributed to the cocoa taste. Most recently, Da Costa *et al.* identified ten diketopiperzines in dry aged and stewed beef and provided sensory data (*1*, *2*). In addition it was observed that longer cooking times led to the generation of more diketopiperazines and that these compounds gave different sensory effects at different concentrations.

In this paper experimental work was devoted to diketopiperazines containing methionine. The presence of a sulfur atom in this type of molecule could add to the enhancement of its organoleptic properties. Thus, eight analogues were synthesized and evaluated organoleptically. In addition, cyclic dipeptide rich sources in nature were identified. Beef extracts and autolyzed yeasts were found to be particularly interesting (Table I). Autolyzed yeasts consist of concentrations of yeast cells that are allowed to die so that the yeasts' digestive enzymes break their proteins down into simpler compounds. They are produced on a commercial scale by the addition of sodium chloride to a suspension of yeast making the solution hypertonic (high osmotic pressure), which leads to the cells shriveling up. This triggers autolysis, in which the yeast self-destructs. The dying yeast cells are then heated to complete their breakdown, after which the husks are separated. These autolyzed yeasts are used in food products such as Marmite™, Bovril™, Vegamite™ and Oxo™.

Liquid/liquid extraction of a variety of autolyzed yeast followed by GC-MS analysis did indeed indicate some to be rich sources of cyclic dipeptides. Methionine containing cyclic dipeptides were also found to be present. Most of these have not previously been reported in nature. namely *cis*-cyclo-(Gly-L-Met), *cis*-cyclo-(L-Ala-L-Met), *cis*-cyclo-(L-Met-L-Val), *cis*-cyclo-(L-Met-L-Met), *cis*-cyclo-(L-Leu-L-Met), *cis*-cyclo-(L-Ile-L-Met), *cis*-cyclo-(L-Met-L-Pro), *cis*-cyclo-(L-Met-L-Phe) (Figure 2). All of these compounds were synthesized and evaluated sensorily.

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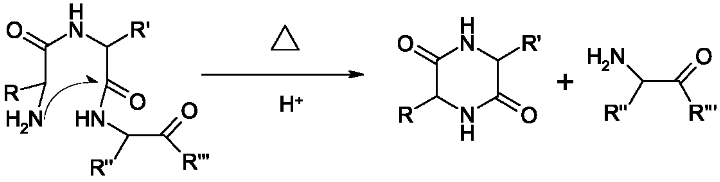

Figure 1. Proposed formation of cyclic dipeptides (11)

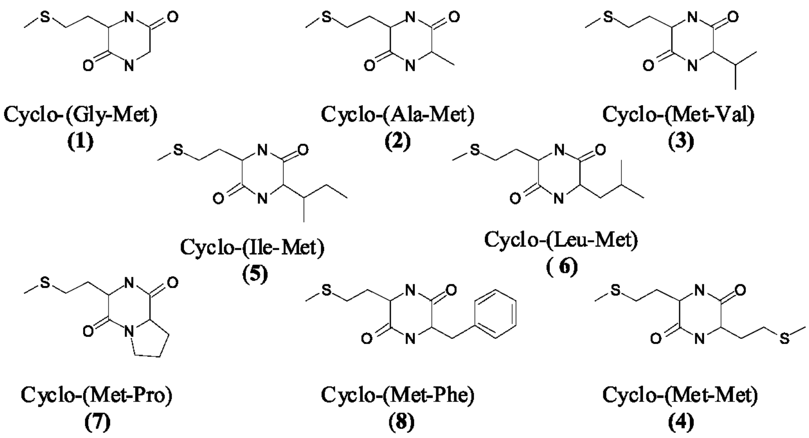
Cyclo-(Gly-Met)
(1)
Cyclo-(Ala-Met)
(2)
Cyclo-(Met-Val)
(3)
Cyclo-(Ile-Met)
(5)
Cyclo-(Leu-Met)
(6)
Cyclo-(Met-Pro)
(7)
Cyclo-(Met-Phe)
(8)
Cyclo-(Met-Met)
(4)

Figure 2. Methionine containing cyclic dipeptides

Table I. Cyclic dipeptide retention order and occurrence in nature

Cyclic Dipeptide	*MW*	*Occurrence in Nature*
cis-Cyclo-(Gly-L-Met)	188	Yeast extract
cis-Cyclo-(L-Ala-L-Met)	202	Yeast extract
cis-Cyclo-(L-Met-L-Val)	230	Yeast extract, Beef extract
cis-Cyclo-(L-Leu-L-Met)	244	Yeast extract
cis-Cyclo-(L-Ile-L-Met)	244	Yeast extract, Beef extract
cis-Cyclo-(L-Met-L-Pro)	228	Yeast extract, Beef extract, Beer
cis-Cyclo-(L-Met-L-Met)	262	Not yet found
cis-Cyclo-(L-Met-L-Phe)	278	Yeast extract

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Materials and Methods

Chemicals

The following reagents were obtained from commercial sources (Sigma-Aldrich, St. Louis, MO, USA): *O*-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), acetic acid, acetonitrile (ACN), N,N-dimethylformamide (DMF), toluene, methanol (MeOH), dichloromethane (DCM), ethyl acetate (EtOAc), triethylamine (TEA), Merrifield resin, N,N-diisopropylcarbodiimide (DIC), potassium fluoride (KF), and N-tert-butoxycarbonyl (Boc) protected amino acids. Note that all amino acids used started in the L-configuration.

Sample Preparation

Yeast autolyzed powder, Maxovar extra Rye-C (30 g) was dissolved in 800 mL of distilled water and transferred to a separating funnel. It was twice extracted with 400 mL of dichloromethane. Centrifugation with cooling was employed to reduce emulsion formation and aid separation. The extracts were combined, dried over anhydrous sodium sulfate, filtered and reduced to 2 mL using a Zymark turbovporator (Caliper LifeSciences, Hopkinton, MA, USA).

Gas-Chromatography

The liquid/liquid extract was analyzed using a HP6890 GC with 50 m non-polar OV-1 capillary column with split ratio of 40:1, flow rate of 1.0 mL/min, temperature program of 40 °C to 270 °C at 2 °C/min with a hold at 270 °C of 10 mins. Analyses were also carried out on a polar phase, 50m CPWax capillary column with a split ratio of 40:1, flow rate of 1.0 mL/min, and a temperature program of 60 °C held for 10 mins then 60 °C to 220 °C at 2 °C/min and held for 20 mins.

GC-Mass Spectrometry and NMR

The chromatographic conditions were the same as described for GC analysis. Micromass Autospec® high resolution, double-focusing, magnetic sector spectrometer, scanning from m/z 450 to m/z 33 at 0.3 secs per decade. MS library identification was achieved using in-house and commercial libraries such as Wiley 7 and NIST. Linear retention indices (LRI) were derived using a series of n-alkanes. Compounds were confirmed using a 500 MHz Bruker 500 Avance NMR spectrometer. Proton and carbon-13 experiments were run to confirm structures.

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Synthesis of Cyclic Dipeptides

A typical synthesis (*12–17*) example was cyclo-(L-Leu-L-Met) which was prepared via solid phase synthesis (Figure 3). Boc-leucine attached Merrifield resin was treated with TFA/DCM solution to remove the protecting group, followed by neutralization with base TEA. Then Boc-protected methionine was coupled to the resin in DMF to form the linear dipeptide on resin. DIC (N, N-diisopropylcarbodiimide) and HOBt were used as coupling reagents. Three equivalents of Boc-Met-OH and coupling reagents were used to drive the reaction to completion. Again, TFA/DCM was used to remove the Boc group of the newly formed dipeptide. In the last step, cyclization occurred in the presence of acetic acid to form the desired cyclo-(L-Leu-L-Met). The crude product had a purity higher than 90% and one simple recrystalization afforded the pure product.

cis-Cyclo-(Gly-L-Met), (**1**): $C_7H_{12}N_2O_2S_1$ (MW 188), ^{1}H NMR ($CDCl_3$) δ: 1.95-2.09 (m, 2H), 2.00 (s, 3H), 2.54 (t, 2H, J = 7.46 Hz), 3.75-3.86 (m, 2H), 3.93-3.97 (m, 1H), 7.49 (s, 1H), 7.75 (s, 1H); EI-MS m/z 188, M$^+$(10), 127 (13), 114 (100), 99 (19), 85 (12), 61 (18), 56 (27), 55 (27).

cis-Cyclo-(L-Ala-L-Met), (**2**): $C_8H_{14}N_2O_2S_1$ (MW 202), ^{1}H NMR ($CDCl_3$) δ: 1.38 (d, 3H, J = 7.00 Hz), 1.93-1.99 (m, 1H), 2.01 (s, 3H), 2.09-2.16 (m, 1H), 2.56 (t, 2H, J = 7.36 Hz), 3.94 (d, 1H, J = 13.91 Hz, of d, J = 6.94 Hz), 4.00 (t, 1H, J = 5.72 Hz), 7.33 (s, 1H), 7.44 (s, 1H); EI-MS m/z 202, M$^+$(30), 141 (20), 128 (100), 113 (66), 61 (24), 56 (19), 55 (12), 44 (40).

cis-Cyclo-(L-Met-L-Val), (**3**): $C_{10}H_{18}N_2O_2S_1$ (MW 230), ^{1}H NMR ($CDCl_3$) δ: 0.84 (d, 3H, J = 6.76 Hz), 0.95 (d, 3H, J = 7.08 Hz), 1.82-1.88 (m, 1H), 1.97-2.02 (m, 1H), 2.03 (s, 3H), 2.12- 2.17 (m, 1H), 2.53-2.58 (m, 2H), 3.68 (m, 1H) 3.93-3.95 (m, 1H), 8.05 (s, 1H), 8.18 (s, 1H); EI-MS m/z 230, M$^+$(26), 169 (25), 156 (81), 141 (13), 113 (100), 99 (7), 85 (14), 72 (18), 61 (40), 56 (26), 41 (14).

cis-Cyclo-(L-Met-L-Met), (**4**): $C_{10}H_{18}N_2O_2S_2$ (MW 262), ^{1}H NMR (d^6-DMSO) δ: 1.89 (m, 2H), 1.98 (m, 2H), 2.02 (m, 6H), 2.53 (m, 4H), 3.94 (m, 2H), 8.21 (m, 2H); EI-MS m/z 262, M$^+$(39), 201 (11), 188 (45), 173 (10), 153 (25), 140 (38), 127 (34), 114 (100), 61 (90), 56 (40).

cis-Cyclo-(L-Leu-L-Met), (**5**): $C_{11}H_{20}N_2O_2S_1$ (MW 244), ^{1}H NMR ($CDCl_3$) δ: 0.85-0.90 (2d, 6H, J = 6.56, and 6.62 Hz), 1.44-1.50 (m, 1H), 1.58-1.64 (m, 1H), 1.78-1.91 (m, 2H), 1.95-2.02 (m, 1H), 2.04 (s, 3H), 2.55 (t, 2H, J = 7.58 Hz), 3.77 (t, 1H), 3.91 (t, 1H), 8.19 (s, 1H), 8.20 (s, 1H); EI-MS m/z 244, M$^+$(29), 183 (34), 170 (100), 155 (20), 114 (60), 113 (73),85 (14), 61 (40), 57 (21), 56 (22), 55 (22).

cis-Cyclo-(L-Ile-L-Met), (**6**): $C_{11}H_{20}N_2O_2S_1$ (MW 244), ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, J = 7.42 Hz), 0.94 (d, 3H, J = 7.05 Hz), 1.16-1.26 (m, 1H), 1.39-1.47 (m, 1H), 1.83-1.92 (m, 2H), 2.01-2.08 (m, 1H), 2.05 (s, 3H), 2.53-2.60 (m, 2H), 3.74 (d, 1H, J = 1.26 Hz), 3.94 (m, 1H), 8.02 (s, 1H), 8.17 (s, 1H); EI-MS m/z 244, M$^+$(27), 183 (30), 170 (83), 155 (12), 139 (8), 127 (7), 114 (35), 113 (100), 99 (10), 85 (15), 69 (11), 61 (39), 57 (21), 56 (22), 41 (21).

cis-Cyclo-(L-Met-L-Pro), (**7**): $C_{10}H_{16}N_2O_2S_1$ (MW 228), ^{1}H NMR ($CDCl_3$) δ: 1.85-2.12 (4H, m), 2.13 (3H, s), 2.32-2.45 (2H, m), 2.69 (2H, t, J=7.02 Hz), 3.51-3.65 (2H, m), 4.11 (1H, t, J=8.2Hz), 4.21 (1H, t, J=5.64 Hz), 6.78 (1H, s);

EI-MS m/z 228, M^+(41), 180 (2), 167 (31), 154 (100), 139 (16), 125 (4), 70 (50), 61 (10), 56 (8), 41 (12).

cis-Cyclo-(L-Met-L-Phe), (**8**): $C_{14}H_{18}N_2O_2S_1$ (MW 278), ^{1}H NMR ($CDCl_3$) δ: 0.95-1.01 (m, 1H), 1.28-1.30 (m, 1H), 1.85-1.90 (m, 2H), 1.87 (s, 3H), 2.82-2.86 (m, 1H), 3.13-3.18 (m, 1H), 3.70-3.73 (m, 1H), 4.19-4.22 (m, 1H), 7.14-7.17 (m, 2H), 7.19-7.23 (m, 1H), 7.25-7.29 (m, 2H), 8.07 (s, 1H), 8.20 (s, 1H); EI-MS m/z 278, M^+(25), 217 (7), 204 (35), 187 (6), 159 (7), 120 (8), 113 (100), 91 (59), 85 (10), 61 (45), 57 (13), 56 (14).

Sensory Evaluation

The cyclic dipeptides were prepared for evaluation in a 0.30% salt water solution as the tasting media. Some of the cyclic dipeptides had solubility issues in water alone, hence the addition of alcohol and the need to warm to achieve homogeneity. Each sample was set up for taste evaluations at levels ranging from 10 ppm to 1000 ppm depending on the taste profile of the peptide. The samples were evaluated by a trained technical panel, typically consisting of five flavorists, using standard flavor descriptors. The evaluations began with tasting the weaker dilutions first and working up to the stronger dilutions. The group comments were recorded and a final descriptor rating given (Table II). Note that all cyclic dipeptides were made up initially at the lower concentrations and only the range where taste effects were observed are reported in the table. Extreme ends of the scale either gave no flavor or just continued bitterness.

Results

From the GC-MS chromatogram of the yeast extract it was observed that many cyclic dipeptides were present in the sample including methionine containing cyclic dipeptides (Figure 4). Additionally an order of elution for these compounds could be generated using alkanes on a non-polar column (Table III). Note that many cyclic dipeptides are too polar or high molecular weight to elute on a standard 50m capillary polar column.

In addition to the methionine cyclic dipeptides found in autolyzed yeast, several other cyclic dipeptides were found, that included many previously reported in the literature. Most were synthesized and confirmed by mass spectra and LRI data (Table III).

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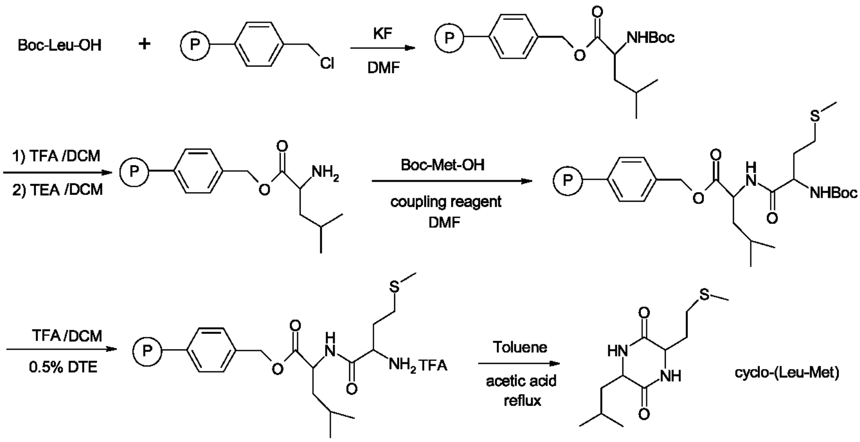
Boc-Leu-OH
+
P
Cl
KF
DMF
NHBoc
O
1) TFA /DCM
2) TEA /DCM
NH2
Boc-Met-OH
coupling reagent
DMF
H
N
S
TFA /DCM
0.5% DTE
NH2 TFA
Toluene
acetic acid
reflux
HN
NH
cyclo-(Leu-Met)

Figure 3. Synthesis route for cyclic dipeptides

Table II. Sensory evaluation of cyclic dipeptides

Cyclic Dipeptide	*Tasting solution*	*Conc. (ppm)*	*Taste Comments*
Cyclo-(L-Ala-L-Met)	0.30% salt water	1000	Creamy cheese, milky
Cyclo-(L-Ala-L-Met)	0.30% salt water	400	Creamy, milky
Cyclo-(L-Ala-L-Met)	0.30% salt water	200	Creamy, milky
Cyclo-(Gly-L-Met)	0.30% salt water	1000	Bitter, rotten, garbage
Cyclo-(Gly-L-Met)	0.30% salt water	400	Creamy, cheesy, melted cheese
Cyclo-(Gly-L-Met)	0.30% salt water	200	Vegetable, creamy
Cyclo-(L-Met-L-Pro)	0.30% salt water	50	Bitter, chalky
Cyclo-(L-Met-L-Pro)	0.30% salt water	10	Bitter, chalky
Cyclo-(L-Ile-L-Met)	Water	100	Creamy, vegetable, celery
Cyclo-(L-Met-L-Phe)	0.5% EtOH/water	20	Creamy, vegetative, metallic
Cyclo-(L-Met-L-Val)	1% EtOH/water	50	Vegetative, metallic, creamy
Cyclo-(L-Leu-L-Met)	1% EtOH/water	100	Weak, little taste
Cyclo-(L-Leu-L-Met)	0.30% salt water	200	Weak, vegetative, creamy, metallic

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Table III. Cyclic dipeptides in autolyzed yeast extract[a]

	Cyclic Dipeptide	*LRI*	*Conc. (ppm)*	*Confirmation*
1	*cis*-Cyclo-(L-Ala-L-Val)	1580	2.5	RI, MS
2	*cis*-Cyclo-(L-Ala-L-Pro)	1651	31.5	RI, MS
3	*trans*-Cyclo-(Ala-Leu)	1673	4.1	MS
4	*cis*-Cyclo-(L-Ala-L-Leu)	1680	10.4	RI, MS
5	*cis*-Cyclo-(L-Val-L-Val)	1687	12.6	RI, MS
6	*cis*-Cyclo-(L-Pro-L-Val)	1767	120.2	RI, MS
7	*cis*-Cyclo-(L-iLe-L-Pro)	1871	29.5	RI, MS
8	*cis*-Cyclo-(L-iLe-L-Leu)	1872	16.3	RI, MS
9	*trans*-Cyclo-(iLe-Pro)	1874	10.2	MS
10	*cis*-Cyclo-(L-Leu-L-Pro)	1876	19.2	RI, MS
11	*cis*-Cyclo-(L-Ala-L-Met)[b]	1884	0.02	RI, MS
12	*cis*-Cyclo-(Gly-L-Met)[b]	1924	0.09	RI, MS
13	*cis*-Cyclo-(L-Met-L-Val)[b]	2023	3.9	RI, MS
14	*cis*-Cyclo-(L-Ala-L-Phe)	2030	4.2	RI, MS
15	*cis*-Cyclo-(Gly-L-Phe)	2036	3.9	RI, MS
16	*cis*-Cyclo-(L-Leu-L-Met)[b]	2130	4.1	RI, MS
17	*cis*-Cyclo-(L-Met-L-Pro)[b]	2168	60.1	RI, MS
18	*cis*-Cyclo-(L-Ile-L-Met)[b]	2157	11.5	RI, MS
19	*cis*-Cyclo-(L-Phe-L-Val)	2162	24.3	RI, MS
20	*cis*-Cyclo-(L-iLe-L-Phe)	2249	0.01	RI, MS
21	*cis*-Cyclo-(L-Leu-L-Phe)	2260	49.4	RI, MS
22	*trans*-Cyclo-(iLe-Phe)	2271	65.2	MS
23	*cis*-Cyclo-(L-Phe-L-Pro)	2274	133.4	RI, MS
24	*cis*-Cyclo-(L-Met-L-Phe)[b]	2559	2.8	RI, MS
25	*cis*-Cyclo-(L-Pro-L-Tyr)	2632	8.7	RI, MS
26	*cis*-Cyclo-(L-Phe-L-Phe)	2798	13.1	RI, MS
27	*cis*-Cyclo-(L-Leu-L-Trp)	2902	2.0	RI, MS
28	*cis*-Cyclo-(L-Pro-L-Trp)	2907	8.2	RI, MS

[a] Linear retention indices (LRI) derived using n-alkanes. [b] Sulfur containing cyclic dipeptides.

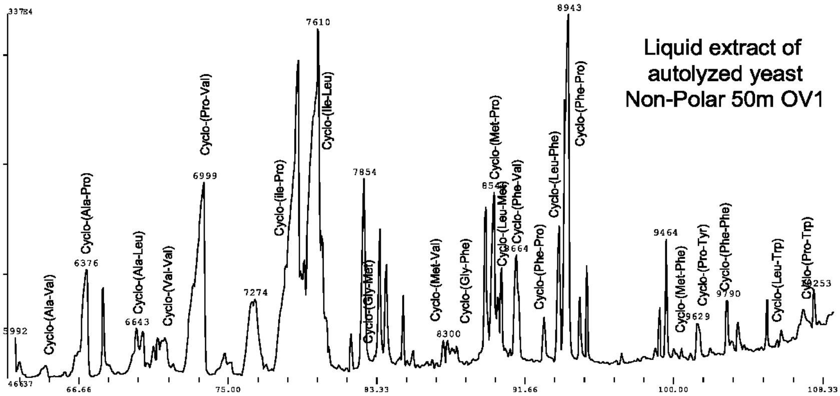

Figure 4. GC-MS of extract of autolyzed yeast showing cyclic dipeptides

Conclusions

Over forty cyclic dipeptides have been reported previously in the literature, having been synthesized and often organoleptically evaluated. Sulfur containing cyclic dipeptides, such as those containing methionine, are of particular interest as they are potentially more interesting organoleptically. In this paper seven out of eight methionine cyclic dipeptides were reported for the first time. In addition flavor evaluations were conducted at different concentrations to give optimum taste effects. Most of the cyclic dipeptides occurred in the autolyzed yeast well below their optimum taste effect concentrations. Thus their contribution to the overall yeast flavor was negligible. Their taste effects were predominantly described as bitter, creamy, milky and vegetative. This last descriptor is more expected of a sulfur containing molecule and not previously described with other cyclic dipeptides. Seven of these methionine containing cyclic dipeptides were detected in food ingredients such as autolyzed yeast. No doubt in the future this sub-class of compounds will be found in more foods and natural products.

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Chapter 12

Control of Color Formation by Ionic Species in Non-Enzymic Browning Reactions

George P. Rizzi*

**Miami University, Department of Chemistry and Biochemistry,
Middletown Campus, Middletown, OH 45042
*georgerizzi@yahoo.com**

Control of visual color production in Maillard reactions was obtained by adding ionic species to model systems consisting of various monosaccharides and amino acids in a neutral aqueous buffer at 100°C. Addition of Na, K, Mg or Ca ions or choline chloride led to increased browning measured by absorbance at 420 nm. Bidentate anions like phosphates, carboxylates and tetraborate also led to enhanced browning as a consequence of their bifunctional structures. A reduction in browning was observed by addition of an ammonium salt to the sugar/amino acid reactions.

Food color can be a crucial determinant of consumer acceptance, and for many processed foods the desired colors are the result of thermally-induced Maillard reactions. Therefore it is of both practical and academic interest to identify catalytic species which can control Maillard browning under approximately neutral conditions. In its initial stage the Maillard reaction consists of a series of ionic processes in which varying ratios of H^+ and OH^- ions can serve as catalysts. Consequently, one ought to obtain similar catalytic control of Maillard browning at neutral pH by supplementing the water-derived ions with other cations and anions.

Cation addition has been shown to influence the course of the Maillard reactions but few studies have related this factor to the control of visual color (*1*). Kato et al. (*2*) reported that iron (II) and iron (III) salts promoted browning in a glucose/ovalbumin system and, more recently, Kwak and Lim (*3*) reported color altering effects caused by six metal ions including those of non-transition

In Controlling Maillard Pathways To Generate Flavors; Mottram, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

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metal sodium, magnesium, calcium and zinc. In food systems containing lipids polyvalent metal ions like iron (II)/iron (III) are probably not viable for practical control of color due to their high propensity to promote lipid autoxidation and formation of off-flavors. Despite this complication, hexanal a common off-flavor compound formed by lipid autoxidation, did lead to inhibition of browning in a glucose/phenylalanine model system containing iron (II) (*4*). Organic cations like amine salts and quaternary ammonium ions may also exhibit control of Maillard browning.

Anionic species, especially phosphates have long been recognized for their ability to influence the course of Maillard reactions, but again few studies have been related to control of pigment formation. Bell (*5*) reviewed earlier studies on buffer effects on pigmentation and confirmed that browning was enhanced in phosphate buffers of increasing concentration. Phosphate and similar bidentate ions offer unique catalytic potential by virtue of their bifunctional structure which allows them to participate in various proton transfer mechanisms. Similar effects have been observed with carboxylates like acetate, hydrogen succinate and citrate ions; however, citrate appeared to diminish browning under relatively mild conditions. Proton transfer mechanisms have been invoked to explain phosphate catalysis in Maillard reactions at various stages including protein glycation (*6*), Amadori rearrangement (*7*) and carbohydrate degradation (*8*). It is likely that a combination of these processes is involved in Maillard pigment formation. Alkyl phosphates, i.e., sugar phosphates occur naturally in foods and are also known to participate catalytically in the Maillard reaction (*9*). In addition, the decomposition of sugar phosphates affords catalytically active inorganic phosphate, reactive intermediates and important sugar-derived flavor volatiles. Borates, e.g., sodium tetraborate (borax) have been employed as food preservatives but had not until recently been associated with Maillard browning (*10*).

It is the purpose of this report to review the results of recent studies on the effects of ionic catalysts on the formation of visible color in the Maillard reaction.

Materials and Methods

Chemicals used were commercial materials of analytical grade. D-sugars were employed except for L-rhamnose. Maillard systems for investigation of cationic and anionic effects consisted usually of aqueous [2, 2-bis (hydroxymethyl)-2, 2′, 2″-nitrilotriethanol] “bis-tris”/HCl buffer (0.10 M, pH 7.26), containing ribose (C_0 = 0.10 M), glycine (C_0 = 0.04 M) and various test substances. Mixtures were heated at reflux, ca. 100°C for 0 to 160 min. and at various times (t) aliquots were withdrawn using a pre-chilled hypodermic syringe and immediately diluted 1:3 or 1:4 with water at 23°C to quench the reactions. The diluted aliquots were quickly analyzed in 1 cm cuvettes using a computer-interfaced UV/VIS spectrophotometer to provide absorbance values (A) measured at 420 nm (vs. pure water, A_{420} = 0.00) to quantify the degree of browning. Replicate experiments typically exhibited absorbance precision ranging from ± 3% to ± 20% with the lowest absorbances showing the greatest

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errors. Cationic test substances included metal chloride salts, choline chloride [2-hydroxy-N,N,N-trimethylethanaminium chloride] and the hydrochloride salts of triethylamine and guanidine.

Effects of phosphate on Maillard browning employed a similar experimental procedure using either the bis/tris buffer or a 0.10 M phosphate buffer (pH 7.3) prepared by mixing 0.10 M solutions of Na_2HPO_4 and NaH_2PO_4. Phosphate studies were performed with ribose and xylose and employed β-alanine instead of glycine to minimize the complication of Strecker degradations.

Borate effects on Maillard browning were investigated by adding 0.0092 M sodium tetraborate decahydrate (borax) to 0.10 M monosaccharide/0.036M glycine reactions in 0.10 M bis/tris buffer at 100°C initially at pH 7.2.

Results and Discussion

Cationic Effects

The effects of externally added cations were examined in a ribose/glycine model system in a relatively inert bis/tris buffer. The buffer bis/tris (pKa 6.5) is approximately 15% protonated, i.e, 0.015 M in its cationic form at pH of ca. 7.3. To supplement the cationic buffer metallic chloride salts of Li, Na, K, Rb and Cs were added at 0.04 M to ribose (0.1M)/glycine (0.04M) reactions run in bis/tris buffer at 100°C. Approximately 20% enhancement in browning (A_{420}) was observed for Group I metal ions after 80 min reaction time and no significant differences were observed between metals (*1*). Addition of Group II chlorides of Mg, Ca, Sr and Ba at 0.016 M to comparable model reactions containing ribose (0.10 M) and glycine (0.035 M) led to more extensive browning (Table I). Mechanisms for metal ion effects are still unclear, but "super acid" effects previously invoked for polyvalent cations (*11*) may be responsible for enhanced Maillard reactions. An example of this type of catalysis might be a Group II ion assisted Amadori rearrangement as shown in Figure 1.

A cationic effect may be at least partially responsible for the well known involvement of phospholipids in Maillard reactions. Lecithins (phosphatidyl cholines) contain a quaternary ammonium moiety whose positive charge suggested potential reactivity. When model compound choline chloride (0.045 M) was added to our nominal ribose/glycine/bis-tris buffer system at pH 7.26 at 100°C a 29% increase in browning (A_{420}) vs. a choline-free control was observed after 80 min (*1*). A second control reaction showed that zero browning occurred in the absence of glycine and suggested that reaction with choline probably took place after the initial amine/carbonyl encounter of ribose and glycine. The ability of choline and lecithins to act as alkylating agents may have led to methylation of the initially formed ribose/glycine Schiffs base intermediate thereby providing a supplemental pathway for Maillard browning (*1*).

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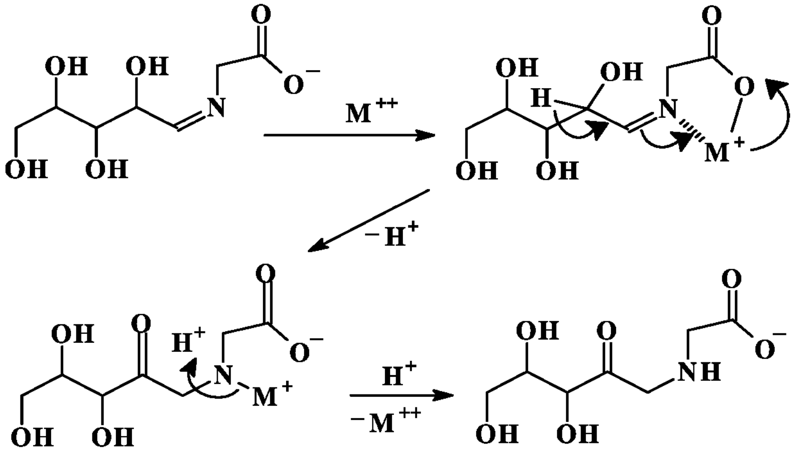

Figure 1. Putative mechanism for divalent metal ion (M^{++}) catalysis of an Amadori rearrangement.

Amines are common constituents of foods and at neutral pH their partially protonated forms are potential cationic catalysts. To test this possibility hydrochloride salts of triethylamine (pKa 11.0) and guanidine (pKa 12.5) were added at 0.04 M to ribose/glycine reactions run in bis/tris buffer (pH 7.26) at 100°C (*1*). Different effects were observed with amines of differing basic strength. With triethylamine hydrochloride a 40-60% reduction in browning (A_{420}) was seen after 80 min, but with guanidine hydrochloride the reverse was true to afford a slight increase (9%) in browning versus a control. In addition, neither amine salt produced any detectable browning with ribose in the absence of glycine. A possible explanation for the observed difference in amine reactivity is the fact that the magnitude of equilibrium constants is known to vary with temperature. From this fact it can be deduced that acidic species like protonated amines could exhibit more effective acidity at 100°C than predicted by their nominal pKa values at 25°C. Thus at 100°C triethylamine hydrochloride is apparently acidic enough to retard the browning potential of glycine by reducing the level of glycinate ion in the ribose/glycine reaction. Evidently the salt of the stronger base guanidine is not sufficiently acidic to affect the concentration of glycinate even at 100°C.

Anionic Effects Produced by Phosphate and Carboxylate Ions

Anionic species, in particular polyatomic anions can exhibit profound effects on Maillard browning. The ability of phosphate ion to accelerate browning has recently been investigated for reactions of β-alanine and a series of pentose and hexose sugars (*8*).

Table I. Effects of Group II Metal Ions on Maillard Browning in pH 7.2 Bis/Tris Buffer at 100°C[a]

Model System [b]	*Metal Ion*	*A_{420}*	*Final pH*
Ribose/Glycine	none	0.335	7.04
	Mg^{+2}	0.424	7.02
	Ca^{+2}	0.519	6.91
	Sr^{+2}	0.397	6.94
	Ba^{+2}	0.429	7.04
Xylose/Glycine	none	0.249	7.22
	Mg^{+2}	0.467	7.09
	Ca^{+2}	0.278	7.01
	Sr^{+2}	0.311	7.17

[a] Source: Adapted from reference (*1*). [b] Reaction time 160 min with 1:4 dilution for A_{420} measurement; C_0 was: sugars, 0.10 M; glycine, 0.035 M; metal chloride salts, 0.016 M.

Table II. Browning of Sugars with or without β-Alanine in 0.10M Bis/Tris and Phosphate Buffers at Initial pH 7.3[a]

Sugar[b]	*Bis-tris*[c]		*phosphate*[c]	
	A_{420}	*Δ pH*	*A_{420}*	*Δ pH*
ribose	0.061	- 0.17[d]	1.17	- 0.86[d]
ribose – no β-alanine	0.003	- 0.16	0.262	- 0.43[d]
xylose	0.050	- 0.09	0.837	- 0.50
xylose- no β-alanine	0.003	0.03	0.150	- 0.28

[a] Source: Adapted from reference (*8*). [b] C_0: sugars, 0.10M; β-alanine, 0.033M. [c] Change in A_{420} and pH after 80min refluxing at ca. 100°C. [d] Changes after 160 min.

The effect of phosphate ion on Maillard browning is exemplified in reactions of ribose and xylose with or without β-alanine present (Table II). In all cases minimal browning and pH change were observed in control reactions performed for 80 min at 100°C in bis/tris buffer initially at pH 7.3. Similar reactions performed in comparable phosphate buffer led to extensive browning, especially with ribose (A_{420} 1.17 in presence of β-ala). Reaction pH decreased during most reactions indicating strong acid formation, but large A_{420} changes caused by phosphate at nearly constant pH (cf. xylose data) indicated that phosphate was acting independently of H^+ or OH^- ions to influence browning. It was significant that considerable browning still occurred in phosphate buffer in the

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absence of β-alanine. Phosphate-induced caramelization, i. e, sugar degradation was confirmed by adding a dicarbonyl trapping agent, 1,2-diaminobenzene, to ribose/phosphate reactions. In this way a series of stable quinoxaline derivatives were formed, which were isolated and served to establish the presence of α-dicarbonyl degradation products of ribose as shown in Figure 2. For example quinoxalins **2** were isolated indicating the presence of α-dicarbonyls [**1** , glyoxal ($R_1 = R_2 = H$) , pyruvaldehyde ($R_1 = H, R_2 = CH_3$), and diacetyl, ($R_1 = R_2 = CH_3$)].

Enhanced browning was also seen in carboxylate based buffers (*8*, *12*), but effects were less than those produced in phosphate. For example, ribose/β-alanine in 0.10M acetate after 80 min at 100°C led to A_{420} 0.15 vs A_{420} 1.17 seen in phosphate under the same conditions. The low pKa of acetic acid prevented effective buffering near neutral pH and the large Δ pH during the reaction (-1.33 pH units) may have influenced pigment formation in the acetate buffer. In a succinate buffer at pH 7.14 better pH control was obtained and a comparable ribose/β-alanine reaction led to more color development and A_{420} of 0.249 under identical reaction conditions. Also, under these conditions a xylose/β-alanine reaction in 0.10 M citrate buffer, initially at pH 7.33, developed A_{420} 0.379 with a pH drop of about 0.8 unit.

The enhancement in Maillard browning caused by phosphate and carboxylate ions is believed to be a result of the bifunctional nature of these ions and their ability to assist proton transfer processes. Based on phosphate results in the absence of amino acids it has been suggested that polyatomic ions can facilitate aldose/ketose equilibration of reducing sugars and dehydration reactions leading to 3-deoxyosones (*8*). The latter compounds and further derived α-dicarbonyls are active precursors of Maillard pigment formation and their formation in the absence of amino acids provides a supplementary pathway for browning that explains the overall visual effects observed.

Anionic Effects Produced by Tetraborate Ion

Recently, it was reported that tetraborate ion could increase the concentration of sugar carbonyl (open-chain form) present in aqueous reducing sugar equilibria (*13*). Since sugar carbonyls are key initial reactants in the Maillard reaction sequence, it followed that borate salts might influence the level of pigment formation. To test the effect of borates, sodium tetraborate was added at 0.0092 M to a series of monosaccharide/glycine reactions in pH 7.2 bis/tris buffer (0.10 M) in which the sugar and glycine were initially 0.10 M and 0.036 M respectively (*10*); for comparison with phosphate similar reactions were performed with 0.0092M disodium phosphate added. In reactions with eight monosaccharides run at 100°C for 80 min all led to enhanced browning (A_{420}) vs controls and, with exception of ribose, all exceeded the levels of pigment formation produced by phosphate. For borate addition the degree of pigment formation decreased in the order: xylose > arabinose > galactose ~ fructose > glucose > ribose > mannose > rhamnose.

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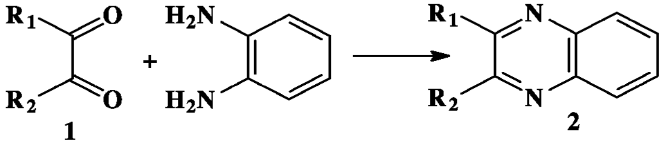

Figure 2. Derivatization of α-dicarbonyl compounds with 1,2-diaminobenzene

The effect of borates on browning was explained by invoking the well known affinity for cis-vicinal glycols to form relatively stable cis-cyclic complexes with tetraborate ions (*10*). Under normal conditions reducing monosaccharides exist in solution mainly as cyclic hemiacetals in equilibrium with tiny amounts of more reactive (open-chain) carbonyl forms. We envisaged that tetraborate ion (formed by hydrolysis of borax) was able to enhance the equilibrium level of reactive carbonyl forms and, thereby, lead to enhanced browning. Sugars that produced more browning with borate, i.e, xylose, arabinose, etc., all have trans-configured adjacent hydroxyl groups in their predominate and unreactive cyclic forms. In these rigid ring structure the trans-configured hydroxyls cannot form cis-borate complexes. But, under equilibrating conditions, sugar ring opening and conformational mobility of adjacent hydroxyl groups takes place to provide 1,2-syn-glycol configurations that are now capable of borate complexation. The formation of cis-borate complexes of open-chain sugars can provide higher concentrations of sugar carbonyls thereby enhancing the overall Maillard reaction. Sugars that give less browning with borate addition, i.e, ribose and mannose have cis-configured C-2/C-3 hydroxyl groups in their rigid cyclic hemiacetal forms, thus allowing them to form Maillard-inactive borate complexes.

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Chapter 13

Effect of Physical Properties of Food Matrices on the Maillard Reaction

A. J. Taylor,[*] L. Sivasundaram, L. Moreau, G. A. Channell, and S. E. Hill

**Division of Food Sciences, University of Nottingham,
Sutton Bonington Campus, Loughborough LE12 5RD,
United Kingdom
[*]Andy.Taylor@nottingham.ac.uk**

Research on the Maillard reaction has provided a good understanding of the chemical pathways leading to those end products that contribute color and flavor to food. There is considerable interest in applying this fundamental knowledge to real foods to reduce undesirable end products, such as acrylamide, or to improve the flavor and color of heated foods. When moving from fundamental experiments, which have mainly used aqueous solutions as the matrix, to foods with lower water contents, the chemistry of the Maillard reaction is affected by the food matrix in a number of ways. Factors of interest are the mobility of the reactants, the temperature profile in different locations in the sample and the effect of phase changes caused by heating. Depending on the type of sample, the matrix might be considered as a single homogenous phase or a multiphase system. Using a real food system (breakfast cereal) and some model systems, the effects of some of these factors have been studied in our laboratory using well controlled processing conditions.

Introduction

Many studies have been carried out on the Maillard reaction in relation to its role in developing color and flavor during thermal processing. The current status was explained well in a review by van Boekel in 2006 (*1*): "It is concluded that the

In Controlling Maillard Pathways To Generate Flavors; Mottram, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

essential elements for predicting the formation of flavor compounds in the Maillard reaction are now established but much more work needs to be done on specific effects such as the amino acid type, the pH, water content and interactions in the food matrix". Since food matrices vary considerably in chemical composition and in their physical properties, research has tended to focus on food types, e.g. starch based foods, or on physical principles that can be applied across different types of matrices (e.g. reactant mobility, water content. glass transitions and phase transitions during processing).

In many thermally processed foods, the initial water content of the food matrices pre-cooking, ranges between 20 and 50% w/w. Examples are many cereal based foods where the initial matrix is a dough or a batter. In these conditions, the matrix consists of a mixture of food components with different degrees of structure. The unprocessed foods can undergo some limited dehydration while waiting for processing or during an initial drying stage and this can have an effect on the Maillard reaction through the concentration of some water soluble solutes. Concentration can occur by the loss of water in the outer regions of a sample, which naturally increases the reactant concentration. There is some evidence from model systems (*2*, *3*) that drying also causes concentration of sugars and amino acids if they are transported on the "solvent front" as the matrix dries. Thus the browning on the outside of a food matrix may be a combination of the surface receiving a higher heat treatment than the interior of the sample, combined with a lower water content and a higher reactant concentration.

In low moisture foods, the water content and reactant availability can be changed if solutes crystallize. This typically happens when certain conditions of relative humidity are reached and the crystallization process can be predicted using various models (*4*). Lactose crystallization has been studied to determine its effect on the glass transition of milk powders (*4*) but systems containing sucrose can also undergo similar transitions (*5*). The situation is complex (*6*) as crystallization can decrease the concentration of reactants while increasing the water content if it releases water that is solubilizing the compound. However, if the compound crystallizes with water of crystallization, this counter effect on water content needs to be considered. Therefore the actual water and reactant concentrations in a heat processed food matrix may be different from those initially set and these can affect the way the Maillard reaction proceeds. Elsewhere in this book, in the chapter on kinetics, van Boekel argues that activity of the Maillard precursors is a more relevant parameter to consider than reactant concentration, and while this is an attractive approach, the determination of compound activity is much more difficult to achieve, compared to a determination of concentration.

When low moisture, starch-containing doughs or mixtures are heat processed, the changes that occur as temperature and moisture content change, can best be followed using a state diagram (Figure 1). By plotting the water content and temperature, the conditions that a sample experiences during processing can be mapped (*7*). Most starch products need to go through the melting temperature to enter into the glassy state on cooling as shown in Figure 1.

Measuring rubber-glass transitions can be accomplished using techniques like Differential Scanning Calorimetry (DSC) and there are several publications that monitor the Maillard reaction in the glassy and rubbery states by adding Maillard

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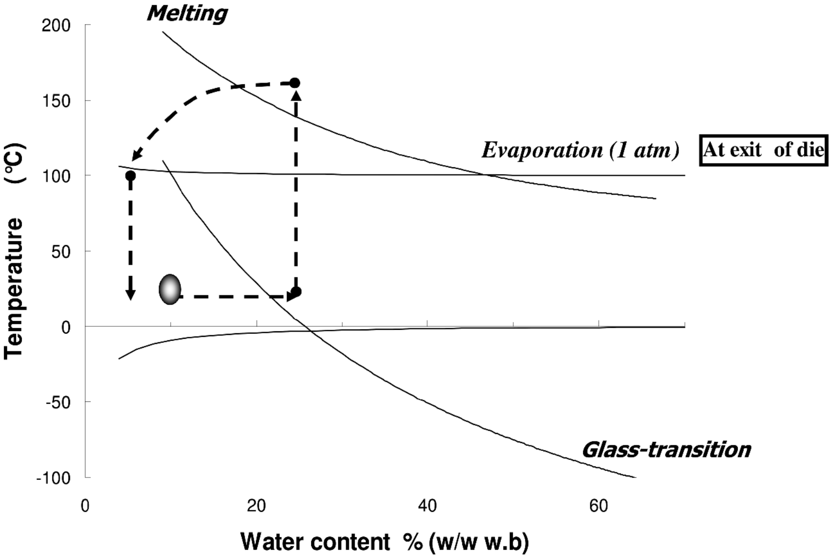

Figure 1. State diagram showing the water content-temperature "journey" for extrusion of starch. The large dot marks the start of the process with starch at about 10% water content. This is hydrated to 25% in the first stage of the barrel then heated from ambient to 150°C in the second stage of the extruder. At this point, the sample passes through the melting temperature of starch and then cools back as it goes through the cooler sections of the extruder barrel and finally is crash-cooled by evaporative cooling as it exits the extruder die.

reactants to matrices with known physical properties (*8*, *9*). During heat processing of foods, the sample spends a short time in the rubbery state whereas, during storage of processed foods, the sample spends a long time in the glassy state. Researchers have tended to focus on the storage condition. Thus browning at 25°C in model systems with different glass transition temperatures (*10*) showed that "at constant water activity, rate constants were higher in systems with lower glass transition temperatures". Other researchers used higher temperatures (40 to 70 °C) to study browning so they could work below and above glass transition temperatures to establish the differences in the rubbery and glassy states (*11*, *12*). In storage studies, other factors, such as retrogradation (rearrangements in starch structure) and the free volume of the system (a measurement of the degree of porosity of the matrix) can also be significant (*10*, *13*).

If a sample is in the glassy state, this signifies that mobility of molecules is decreased compared to the rubbery state. For large biopolymers like starch, the mobility is seriously decreased and the molecules are "frozen" in the form they were in just prior to entering the glassy state. For small molecules like water, sugars and amino acids, the effect is less severe and, rather than going through a step-type change in their mobility, the change is flatter and less pronounced. Studies on the diffusion of small molecules around glass transition temperatures demonstrated some anomalies (*14*). Therefore glass transition measurements

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provide information on the state of the macromolecules but do not always give precise information on the mobility of the small molecules.

Several mechanisms have been proposed to explain why changing the glass transition temperature affects the Maillard reactions and the formation of end products like color, acrylamide or flavor. One hypothesis suggests that when the glass transition temperature of a sample is decreased by addition of a plasticizer (a compound that changes the glass transition temperature), the sample will spend more time in the rubbery state during heating. In the rubbery state, reactant mobility is higher, and this will increase the rate/extent of the Maillard reaction. Another hypothesis proposes that, due to the hygroscopic nature of plasticizers like sugars and salts, water loss during heating is delayed and the reactants are therefore more mobile/reactive in the presence of hygroscopic solutes. There is also the dilemma that a typical Maillard pathway goes through some dehydration steps then through hydrolysis steps later in the reaction sequence. In these situations, water can exert an indirect effect on the reaction through reactant mobility in the matrix as well as a direct effect on the reaction by the level of water present and therefore the relative degree of dehydration/hydrolysis that can occur. The sensitivity of these reactions to moisture content may be a way to control the pathways during processing. In this paper, we report research designed to apply fundamental principles to food, or model systems designed to mimic real foods, as well as systems that attempt to control processing conditions, so that the time/temperature/humidity conditions are constant and the data obtained reflect the chemistry that is occurring rather than any variability in processing conditions.

Measuring the end products of the Maillard reaction in these matrices can be relatively easy, for example by measuring color formation in the matrix but measurement of the flavor compounds produced by the Maillard reaction requires significant analytical work. Typically, multiple samples are heated under different conditions (e.g. time, temperature, initial water content) and then extracted and analyzed using GC-MS or LC-MS. An alternative is to use on line MS to monitor volatile end products, which gives real time information as well as allowing some Maillard intermediates to be monitored. The response of the system to rapid changes in processing parameters can also be studied (*15*) and data can be acquired relatively quickly. The downside of real time monitoring is the difficulty of assigning compounds to the ions monitored in the MS although this can be achieved to some degree using complementary techniques like GC with parallel detection using EI-MS to identify the compounds and PTR or APCI ionization to determine which ions are formed from those compounds (*16*, *17*). Using labeled precursors can also aid identification in some cases (*18*).

The preceding paragraphs describe the issues that need to be considered when studying the Maillard reaction in thermally processed foods. The ultimate aim of all these studies is to gain a better understanding of the control points in the systems and then try and find ways to apply them in real foods so as to avoid the formation of undesirable compounds and favor the production of desirable compounds. The following sections give some examples of this kind of approach to investigate the effects of the physical properties of the food matrix on the Maillard reaction.

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Materials and Methods

Browning in Milk Powder Systems

Studies on the physical state of the components of milk powder and whole milk powder were carried out as described previously (*19*, *20*). Phase changes were monitored by DSC and FTIR, while formation of flavor was monitored by on-line APCI-MS.

Effect of Salt on Color Formation in Breakfast Cereals

A model breakfast cereal matrix was developed which had very similar properties to a commercial breakfast cereal but allowed easy manipulation of formulation and processing. The effects of salt on water retention during processing and on the plasticization of the matrix were studied using thermogravimetric analysis and DSC (*21*).

Effect of Water on Dehydration/Hydrolysis Reactions in the Maillard Reaction

A Strecker reaction between valine and glucose was carried out by making thin films of reactant on a glass bead support. This allowed rapid heat transfer into the matrix as well as allowing rapid release of volatiles formed from the Strecker reaction. A flow of gas through the system allowed control of humidity in the glass bead reactor and pulses of ^{18}O labeled water were introduced to study the inter-conversion of intermediates in the Strecker pathway (*15*).

Results and Discussion

To investigate the effects of food matrix on crystallization, samples of pure lactose, a mixture of lactose and lysine (3:1 w/w) and skim milk powder were analyzed by DSC and the results are shown in Figure 2 (dwb is water on a dry weight basis). Lactose (trace A) showed a major change in heat flow when it crystallized around 145°C but the glass transition event was accompanied by a minor change in heat flow. Trace B (lactose and lysine) showed a glass transition then a significant change in heat flow above 100°C but not associated with lactose crystallization.

Skim milk powder showed a different behavior with the onset of the exotherm occurring at a high temperature and showing a different shape compared to samples A and B. Lactose and lysine in a 3:1 ratio has been proposed as a model of milk powder but comparison of traces B and C show very different thermal behaviors and suggest that, in terms of matrix thermal properties the two systems are different.

For each of the samples, DSC pans were removed and opened at certain temperatures and the color of the contents scored qualitatively (*19*). Lactose remained as a white powder over the whole heating period, while the lactose:lysine mixture showed initial signs of browning at the maximum endotherm value

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(115°C) and produced a dark brown color at 130°C. Skim milk powder showed a dark brown color at its maximum endotherm value at 140°C and a similar color at 150°C. Thus the differences in thermal behavior are reflected in the browning behavior of the samples. Lactose crystallization was measured in the heated samples from the DSC pans and this confirmed the lack of crystallization in the lactose:lysine and skim milk powder samples.

Experiments were also carried out on dry samples held on the diamond anvil of a reflectance FTIR to monitor chemical changes. Glass transition could be determined by the change in intensities at certain wavelengths. Finally, the temperatures at which volatile Maillard products were formed were measured using APCI-MS and the data combined to show the overall picture (Figure 3).

The experiments above show the importance of using real food matrices to understand their effects on the Maillard reaction. Therefore when studying the effect of salt on the Maillard reaction in breakfast cereals, the first thought was to use the commercial formulation. However, the commercial formulation uses ingredients like wheat, which is known to vary in composition with time (due to respiration and other effects) as well as varying depending on the origin. A model system was developed (consisting of pre-gelatinized maize starch, glucose and a mixture of amino acids) that mimicked the behavior of breakfast cereals in terms of color, volatile and acrylamide production. The model formulation (Table I) allowed consistent batches of products to be prepared over long time periods (12-18 months). The components were mixed with 250 mL water, freeze dried, rehydrated to 15% moisture (wet weight) and then heat processed for times between 0 to 10 min at 230°C.

Observations from commercial breakfast cereal production were that decreasing salt content decreased the color of the finished product. The result was that breakfast cereal products with reduced salt content lacked the usual golden color and were perceived as less attractive by consumers. Two hypotheses were proposed to account for this observation. One was that the presence of salt exerted a hygroscopic effect and retained water during processing so that the Maillard reaction occurred at higher water activities and more Maillard products were formed. The second hypothesis was that salt plasticized the cereal matrix and so the system spent more time in the rubbery state where molecular mobility increased and the result was increased browning.

To test the first hypothesis, model cereal samples were prepared with different levels of salt (Table I) and 20% moisture content and then subjected to thermogravimetric analysis (TGA). The concept was that heating the samples in the TGA would mimic water retention during processing. Figure 4 shows the water loss for samples containing 0, 2.5 or 5% salt as they are heated from ambient temperature to around 200°C. All three samples showed very similar behavior and these data strongly suggested that salt did not exert a hygroscopic effect and, therefore, the first hypothesis could not be supported.

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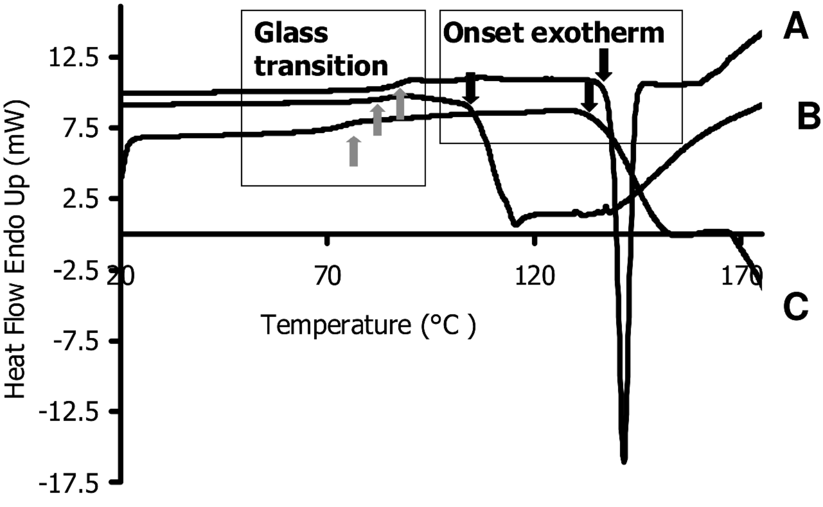

Figure 2. DSC traces from ***A*** *– lactose (1.5% dwb);* ***B*** *Lactose:lysine 3:1 mixture (1.7% dwb) and* ***C*** *skim milk powder (3% dwb). The up arrows indicate glass transitions. The sharp exotherm for lactose indicates crystallization, the exotherms for lactose:lysine and skim milk powder indicate chemical reactivity.*

Table I. Model system composition to study effect of NaCl on browning; salt content was varied to give 0 to 5% dwb NaCl and the amount of maize starch was adjusted to compensate for added salt

Component	*Weight (g)*
Pregelatinized maize starch	49.134- 46.634
Leucine	0.147
Valine	0.054
Alanine	0.032
Glutamic acid	0.516
Glucose	0.983
NaCl	0-2.5

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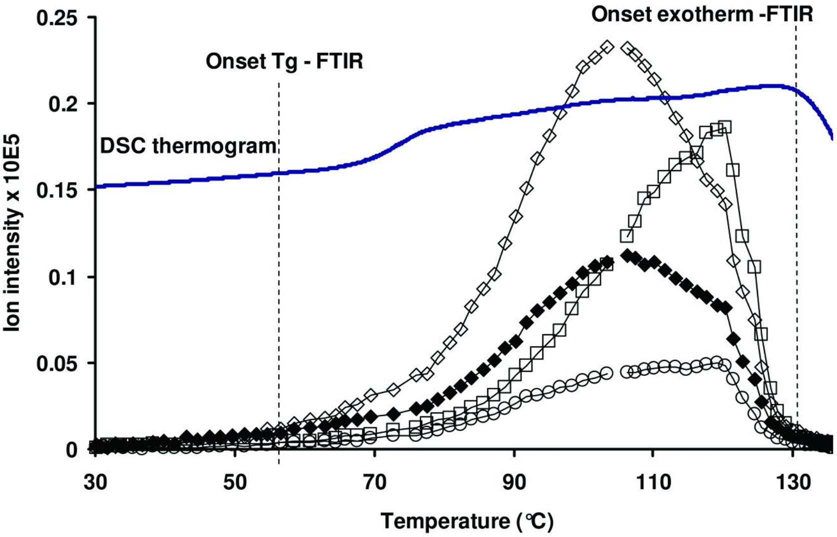

Figure 3. DSC and APCI traces showing relationship between volatile compound formation and physical changes in matrix. Dotted lines show FTIR measurements of T_g and the onset of the exotherm. Volatile compounds were provisionally identified as furfuryl formate/maltol (open circles), furfural (open diamonds), acetylfuran (filled diamonds) and diacetyl (open squares).

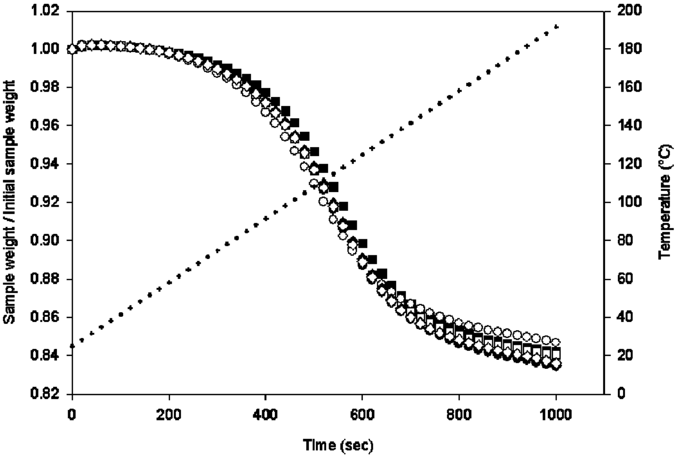

Figure 4. Thermogravimetric analysis of model cereal samples containing 0, 2.5 or 5% salt (denoted by square, circle or diamond markers respectively).

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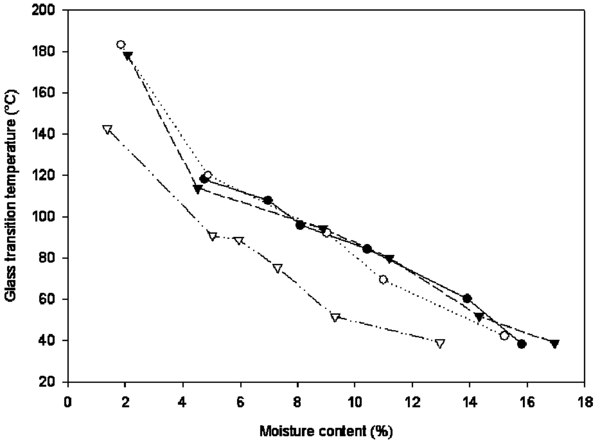

Figure 5. Glass transition temperature in cereal samples as a function of moisture content. The lines close to each other are the samples containing NaCl or KCl or no salt, the other line is the sample with 25% trehalose.

To test the second hypothesis, the glass transition temperature of the model cereal samples was measured using a phase transition analyzer (PTA) rather than DSC. PTA has advantages over DSC in terms of sample size and in its operating cycle which mimic food processes better than the static DSC measurement.

Figure 5 shows that, there is little effect on glass transition temperature (Tg) at the levels of salt used in this experiment. Trehalose was added at high levels to investigate what sort of plasticizer level would be needed to change the Tg significantly. The conclusion from this experiment was that Tg was not significantly affected by salt levels in the range found in regular and low salt breakfast cereals. Neither hypothesis accounted for the decrease in color as salt levels were decreased and indicates that other matrix effects need to be considered and investigated.

Another way to investigate the effect of water on the Maillard reaction uses model systems deposited as thin, low moisture films on a glass bead support. The thin films allow rapid heat transfer so that each part of the system receives the same heat treatment (*15*) and any volatile products are rapidly released into a flow of gas which is fed into an APCI-MS analyzer to monitor the formation of both intermediates and end products. The system chosen for study was a Strecker reaction shown in Figure 6.

The system was chosen for its relative simplicity and because preliminary experiments had established the APCI-MS operating window and the conditions in the film reactor that allowed monitoring of Intermediates 1 and 2 as well as the products of Intermediate 2 breakdown. Aminoacetone is a recognized product of the Strecker reaction but 2-methylpropan-1-amine has not been reported and is included from a consideration of the potential chemical pathways when the tautomers of Intermediate 2 are hydrolyzed. APCI-MS protonates the compounds so Intermediate 1 appears on the ion trace at *m/z* 172, Intermediate 2 at *m/z* 128 and the breakdown products of Intermediate 2 share the same molecular weight

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and give a common ion at *m/z* 74, which is indicative of the overall breakdown of Intermediate 2, irrespective of the relative amounts of each tautomer. The reaction was started by increasing the temperature from 130 to 170°C and, at the same time, the water content of the gas stream was cycled by changing the volume of water injected into the gas stream humidifier. The cycle started with 1 µL/min of water, then changed to zero water, then to 5 µL/min and back to 1 µL/min. The three ions were monitored as these changes took place and are shown schematically in Figure 7.

Interpreting the traces is best done by comparing the relative amounts of the three compounds at different levels of humidity. As humidity decreased from 1 µl/min to 0 µL/min, the level of Intermediate 1 increased as the drier conditions encouraged the dehydration reaction and more of this compound was formed. However the amounts of Intermediate 2 (formed by decarboxylation of Intermediate 1) and the end product (formed by hydrolysis of Intermediate 2) decreased. At 5 µL/min humidity, the formation of all three compounds was decreased suggesting that formation of Intermediate 1 was effectively stopped and therefore, the other two compounds were not formed.

This kind of investigation is useful to give information on the sensitivity of the chemical pathway to humidity levels. However, the limitations of the experimental system need to be considered. Defining humidity levels in gases at higher temperatures falls outside the parameters used at temperatures under 100°C (e.g. water activity) when water is mainly in the liquid phase, and finding a parameter that relates to the amounts of reactants present needs some thought. Preliminary data analysis in our laboratory suggests that expressing the "humidity" as moles of water per minute and comparing the ratio of moles of water to moles of reactant may be a better way to express the relationship between water and reaction rates/amounts.

The system also measures the end product in the gas phase, whereas the food industry is more interested in the amounts retained in food, which, after all, actually deliver food flavor to the consumer rather than expelling it via the factory chimney. In acrylamide formation, we showed a linear relationship between gas phase acrylamide and the amounts of acrylamide found in the solid matrix after processing (*22*) but no such experiments were carried out on the Strecker system. Assigning compounds to ions is also problematical. In the simple Strecker system described here, the ions and compounds can be assigned although measurement of the actual Strecker aldehyde cannot be accomplished due to the predominance of the nitrogen-containing Maillard products in the mass spectrum. However, further research could address these issues and help in developing a system that could yield valuable information about the response of Maillard chemical pathways to factors like humidity, pulsed humidity or addition of other reagents like hydrogen sulfide to mimic their formation from other Maillard pathways.

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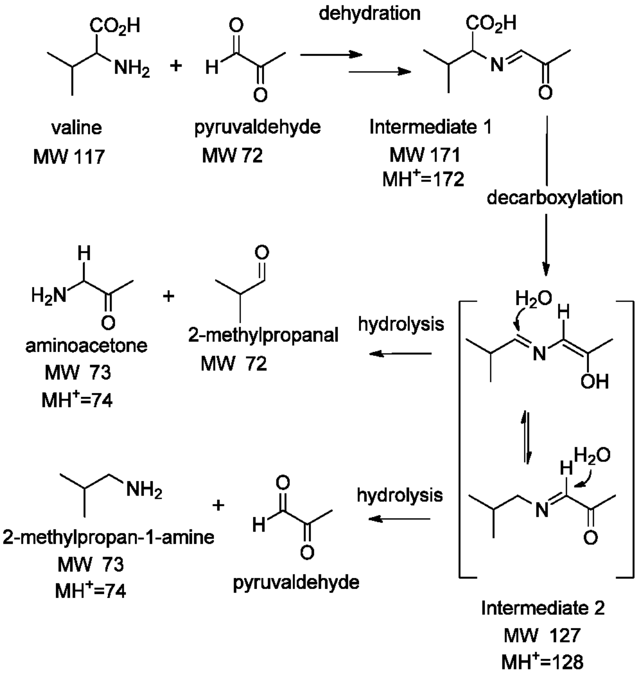
valine
MW 117
pyruvaldehyde
MW 72
dehydration
Intermediate 1
MW 171
MH+=172
decarboxylation
aminoacetone
MW 73
MH+=74
2-methylpropanal
MW 72
hydrolysis
2-methylpropan-1-amine
MW 73
MH+=74
pyruvaldehyde
hydrolysis
Intermediate 2
MW 127
MH+=128

Figure 6. Chemical pathways from valine to the corresponding Strecker aldehyde, 2-methylpropanal. The reactive carbonyl is shown as pyruvaldehyde. The reaction proceeds through a dehydration step, decarboxylation and then a hydrolysis step.

The system was also used to determine whether added water in the gas phase plasticizes the matrix and allows reaction to occur or whether added water acts as a reagent (Figure 8). The film reactor was prepared with leucine and glucose and the formation of 3-methylbutanal monitored (*m/z* 87). A pulse of ^{18}O labeled water was then injected into the gas stream and the presence of ^{18}O labeled methylbutanal (*m/z* 89) monitored. The presence of water in the outflow gas can be monitored by following the protonated water trimer ion ($(H_2O)_3H^+$; *m/z* 55) which was replaced by *m/z* 61 when ^{18}O labeled water was injected, showing that the labeled water had fully displaced the non-labeled water. The ion at *m/z* 89 (corresponding to labeled methylbutanal) showed an increase from baseline which coincided with the introduction of labeled water. This is evidence that water from the gas phase actually penetrates the thin layers of reactants in the film reactor and takes part in the hydrolysis reaction. It may also have a complementary plasticizing action but this cannot be determined from these experiments.

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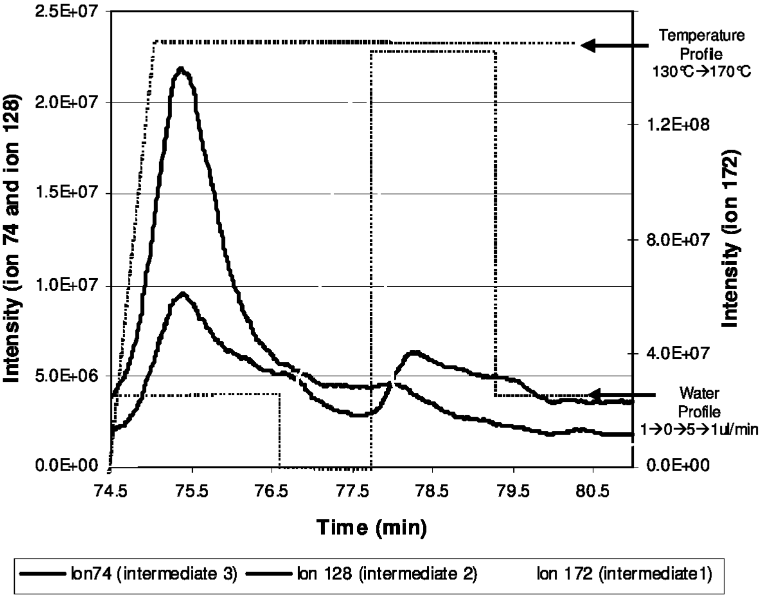

Figure 7. Amounts of Maillard products formed in response to changing humidity levels in the gas stream of the film reactor. Temperature was raised from 130 to 170°C and water profile was varied from 1 to 0 to 5 to 1 μL/min.

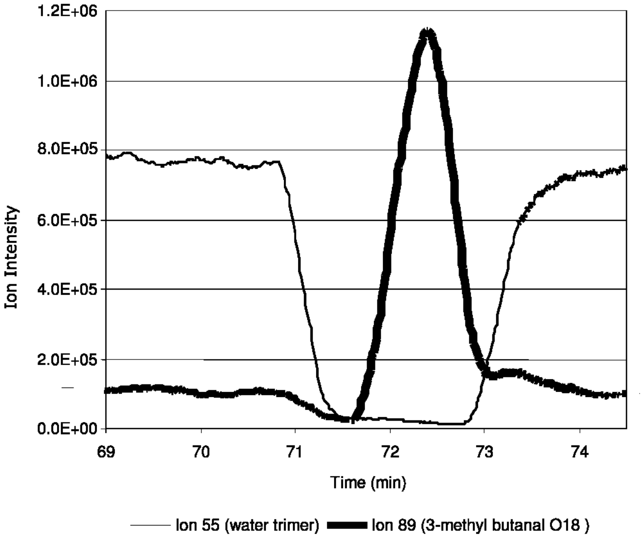

Figure 8. Incorporation of ^{18}O label from water into the Strecker aldehyde, 3-methylbutanal. A reaction producing 3-methylbutanal received a pulse of ^{18}O labeled water between 71 and 73 min and label was incorporated presumably due to the hydrolysis step shown in Figure 5.

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Conclusion

The data presented illustrates how the physical properties of different food matrices can affect the course of the Maillard reaction. There are many interlinked factors involved and studies to unravel these factors require careful experimental design and novel analytical methodologies to make an impact. There is evidence that research groups around the world are now studying the Maillard reaction in complex systems to apply the existing knowledge on chemical pathways and mechanisms to help control flavor and color formation in the manufacture of real food products.

Acknowledgments

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Chapter 14

Control of the Maillard Reaction during the Cooking of Food

Donald S. Mottram*[*] and J. Stephen Elmore

Department of Food and Nutritional Sciences, University of Reading, Whiteknights, Reading RG6 6AP, United Kingdom
[*]d.s.mottram@reading.ac.uk

It was almost 100 years ago when Louis-Camille Maillard discovered the reaction between amino acids and sugars that was subsequently named after him. However, it was another 40 years before its contribution to food flavor was appreciated. Since then many hundreds of compounds derived from the Maillard reaction have been identified in cooked foods. Studies of model Maillard systems have provided understanding of the pathways involved in formation of these compounds. However, model systems never deliver all the sensory characteristics of cooked foods and model systems are much more susceptible to small variation in reaction conditions. In a food the complex mixture of sugar and amino acid precursors, and the presence of structural components and other reactive compounds, provide control of the Maillard reaction so that consistent and characteristic flavor is delivered in that food. This paper reviews some of the interactions that occur in real foods during cooking and thereby provide natural control of Maillard flavor in food.

Introduction

The reaction known as the Maillard reaction, which occurs when reducing sugars are heated with amino acids, peptides and other amino compounds, has been widely studied in food science and, more recently, has been of increasing interest in medicine. It was named after the French chemist Louis-Camille Maillard (1878-1936). He discovered the reaction when he heated glucose with glycine during investigations of the use of polyols as dehydrating agents during the synthesis of peptides (*1*). He noted the formation of orange and brown colors and showed that the reaction was common to all reducing sugars and amino acids. The work was published in a few papers in the early part of the twentieth century, the first in 1912. Little or no interest was shown in the reaction for another 40 years until brown colorations were noticed in dried foods, such as milk powder. Initially described as non-enzymatic browning, the term "Maillard Reaction" was adopted in the early 1950s. The reaction also provided an explanation for the formation of the flavors associated with cooked foods. In recent years the physiological significance of the reaction has been recognized in relation to *in vivo* glycation of proteins and the link to diabetic complications, cardiovascular and other diseases (*2*, *3*). The possibility of mutagenic compounds being formed in the Maillard reaction has also been recognized and particular attention has been directed towards heterocyclic aromatic amines in grilled meat (*4*) and acrylamide (2-propenamide) in fried and oven-cooked potato and cereal products (*5*, *6*).

The first aroma compounds derived from the Maillard reaction to be identified in a food were 2-furanmethanethiol and several alkylpyrazines found in coffee in 1927 by Reichstein and Staudinger in a remarkable piece of analytical chemistry using classical separation and identification techniques (*7*). It was not until the 1960s, and the availability of gas chromatography and mass spectrometry, that significant numbers of aroma compounds were identified in foods.

The discovery of the reaction is rightly attributed to L.-C. Maillard, however, the unraveling of the complex chemistry was the outstanding contribution of the American carbohydrate chemist, John Hodge, who worked at the USDA laboratories in Illinois. In 1953 Hodge drew up a scheme to explain the essential steps leading to the formation of melanoidin pigments (*8*) and to the formation of aroma compounds (*9*). It is noteworthy that some 50 years later, the Hodge scheme still provides the basis for our understanding of the reaction.

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Stages in the Maillard Reaction

The scheme devised by Hodge divides the Maillard reaction into three stages. A simplified version of the basic scheme, illustrating flavor formation, is shown in Figure 1. The reaction is initiated by the condensation of the carbonyl group of a reducing sugar with an amino compound, producing a Schiff base. Acid-catalyzed rearrangement gives a 1,2-enaminol, which is in equilibrium with its keto tautomer, an N-substituted 1-amino-2-deoxyketose, known as an Amadori rearrangement product. Ketosugars, such as fructose, give the Heyns rearrangement product by related pathways. The Maillard reaction has been the subject of many mechanistic studies, usually through investigations of single amino acid and sugar systems. It has been extensively reviewed, e.g. (*2*, *3*, *10*).

The Amadori and Heyns rearrangement products are unstable above ambient temperature. They undergo deamination, dehydration, and fragmentation, giving rise to a mixture of sugar dehydration and fragmentation products containing one or more carbonyl groups, as well as furfurals, furanones and pyranones (Figure 1). These carbonyl compounds react with free amino acids through

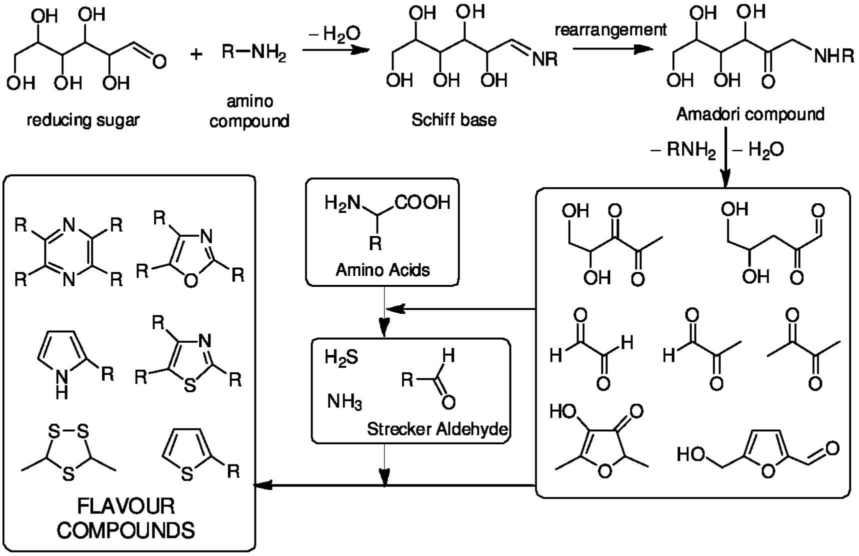

Figure 1. The essential steps of the Maillard reaction leading to the formation of aroma compounds.

Strecker degradation, in which the amino acid is deaminated and decarboxylated, to yield the corresponding Strecker aldehyde. The aldehydes, other carbonyls, furfurals, and furanones obtained from the sugar may contribute to flavor characteristics associated with the Maillard reaction. However, they are also important intermediates for the formation of other aroma compounds.

The products of the initial and intermediate stages of the Maillard reaction are colorless or pale yellow and Hodge attributed color formation to the final stage of the reaction, where condensation between carbonyls (especially aldehydes) and amines occurs to give high molecular mass, colored products known as melanoidins. These have been shown to contain heterocyclic ring systems, such as pyrroles, pyridines, and imidazoles, but their detailed structures are unknown.

The final stage of the Maillard reaction is of great importance for flavor formation when carbonyl compounds react with each other and with amino compounds and amino acid degradation products, such as hydrogen sulfide and ammonia. It is these interactions that lead to the formation of flavor compounds, including important heterocyclics, such as pyrazines, pyrroles, furans, oxazoles, thiazoles and thiophenes.

Study of Maillard Reaction in Model Systems

One of the earliest reports of the Maillard reaction between sugars and amino acids being used for the generation of flavor was the patent of Morton, Akroyd and May in 1960 which used cysteine and ribose to produce meat-like aromas (*11*). Many patents and papers followed, many with the aim of producing flavorings for the food industry that had improved savory and roast characteristics (*12*). The development of some such flavorings is discussed in another chapter

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in this book (*13*). During the next three decades, many novel aroma compounds were identified in cooked foods and, in an attempt to determine their formation pathways, model reactions were carried out between individual amino acids and reducing sugars. Recently we compiled a list of volatile compounds that had been identified in Maillard model systems in 38 papers published between 1985 and 2002 (J.S. Elmore and D.S. Mottram, unpublished). Fifteen amino acids and seven sugars were used in these papers yielding a total of 621 volatile compounds (Table I). This list is not fully inclusive and other relevant papers appeared before and after this period. However, it illustrates the breadth of the research area and the large number of compounds that could contribute to aroma. Many of the compounds are heterocyclic and those containing nitrogen or sulfur have low odor threshold values; consequently only trace quantities are needed to contribute odor characteristics. This is particularly notable for sulfur-containing compounds.

One of the principal routes by which nitrogen is introduced into aroma molecules during the Maillard reaction is via the Strecker reaction (Figure 2). In the deamination of the amino acid by a dicarbonyl compound, an amino ketone is formed, which is relatively unstable and further reacts to give pyrazines, oxazoles and thiazoles, many of which contribute to roasted aromas in foods. Free ammonia may also be released from amino acids, and hydrolysis of cysteine gives rise to ammonia as well as hydrogen sulfide.

Sulfur-containing heterocyclic compounds, such as thiophenes, thiazoles, trithiolanes, thianes, thienothiophenes, and furanthiols and disulfides, play major roles in determining the characteristic aromas of cooked foods. Many of these compounds have extremely low odor threshold values and so make significant contributions to aroma at the part per billion (μg/kg) level or lower. Hydrogen sulfide is a key intermediate in the formation of many heterocyclic sulfur compounds. Figure 3 summarizes the reactions between hydrogen sulfide and other simple intermediates formed in the Maillard reaction.

Meat has higher levels of cysteine, the principal sulfur-containing amino acid, than most other foods and, consequently, sulfur compounds are major contributors to meat aroma. In particular, furans and thiophenes with a thiol group in the 3-position, possess strong meat-like aromas and exceptionally low odor threshold values (*14*) and a number of such compounds have been isolated in the volatiles of cooked meat, including 2-methyl-3-furanthiol and the corresponding disulfide, bis-(2-methyl-3-furanyl) disulfide (*15*, *16*). Disulfides and thiols containing a furan ring have also been found among the volatiles of coffee; however, in coffee those containing the 2-furylmethyl moiety are more abundant than compounds with the 2-methyl-3-furyl moiety (*17*).

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Maillard Reaction in Foods

Studies of the Maillard reaction between individual amino acids and sugars have provided a wealth of understanding on the pathways for formation of aroma compounds and have provided considerable insight into the range of compounds that are produced in the reaction. However, real foods are much more complex and data provided by the models do not reflect the aroma profiles generated in real foods. The free amino acids in foods comprise mixtures of all the common occurring amino acids, in different proportions in the different foods. Similarly, they contain several different sugars. In some high carbohydrate foods such as cereals, sugars may be present in excess compared with free amino acids but in proteinaceous foods, such as meat, the amino acids are in excess over the sugars. Thus competition can occur between amino acids for available sugar or between sugars for available amino acids. Hence, the aroma profile in a food is unlikely to be a simple summation of the profile produced by the reactions of individual precursors. The aroma compounds produced in the Maillard reaction are derived from the interaction of different intermediates and, in a food, these are provided by reactions of mixtures of amino acids and sugars; thus many different interactions will be possible. As a consequence, it is very difficult to predict the course of a Maillard reaction in a food or provide external means of controlling the reaction pathways. However, it is interesting to reflect how much the food composition and structure control the reaction. Each food is very clearly recognizable from the aroma produced during cooking under wide ranges of conditions.

Other reactions occurring during cooking may also provide intermediates for the later stages of the Maillard reactions. Many lipid degradation products contain carbonyl groups and these are able to react alongside sugar-derived carbonyls in the later stages of the Maillard reaction (*18*).

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Competition between Maillard Precursors

The discovery, in 2002, that acrylamide was generated in heated cereal and potato products, led to an upsurge of interest in the Maillard reaction. It was quickly established that the essential precursor for acrylamide was asparagine (*6*) and the pathway by which it is formed involved the formation of a Schiff base from the asparagine and a reducing sugar, and its subsequent decarboxylation, rearrangement and fragmentation, via a mechanism related to Strecker degradation (*19*). However, this reaction on its own cannot predict the rate of formation of acrylamide in a heated food. At elevated temperatures, acrylamide will react with amino and sulfhydryl groups and this needs to be taken into account in predicting yields (*20*, *21*). In foods, other Maillard reactions will take place alongside that of asparagine. These could influence the formation of acrylamide by competing with asparagine for available sugars and, when sugars are limiting, reducing the amount of asparagine reacting. Conversely, the Maillard reaction will provide carbonyls from the breakdown of sugar and these could react with asparagine to give acrylamide and, thence, increase acrylamide yields. The different Maillard reaction pathways that may contribute to acrylamide formation are shown in Figure 4.

Some recent data from our laboratory illustrates the roles that other amino acids play in determining the amount of acrylamide formed in heated potato and cereals. Through changing the agronomic conditions, in particular sulfate content of the soil, for different varieties of wheat and potato, we obtained material that had a wide range of asparagine levels (*22*, *23*). In wheat, sugars were in considerable excess compared with the free amino acids and in the heated wheat flour very good correlation between asparagine content and acrylamide formation was found,

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Table I. Maillard model systems reported in literature between 1985 and 2002, showing reactants used and numbers of volatile compounds produced

Sugars
glucose
fructose
ribose
ribose phosphate
rhamnose
arabinose
sucrose

Amino acids
glycine
isoleucine
lysine
serine
phenylalanine
proline
hydroxyproline
cysteine
methionine
glutathione
threonine
aspartic acid
asparagaine
glutamine
γ-aminobutyric acid

Numbers of volatile compounds	
Pyrazines	59
Pyrroles	104
Pyridines	40
other N-compounds	20
Thiophenes	67
Thiazoles	40
other S-compounds	102
Furans	90
Oxazoles	6
other O-compounds	83
Aromatic hydrocarbons	10
TOTAL	621

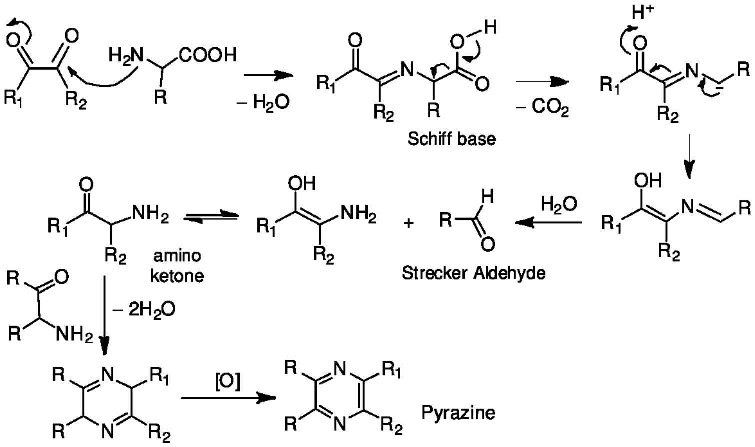

Figure 2. Strecker degradation of amino acids and formation of alkylpyrazines.

indicating that acrylamide could be predicted from the asparagine level in the flour. However, in potato these were not correlated (n=18; r^2=0.23). Sugars are not usually in excess in potatoes, which will affect the asparagine–acrylamide correlation, but acrylamide was not correlated with sugar levels either (r^2=0.42). However, when the ratio of asparagine to total free amino acids was plotted against acrylamide, a good correlation was obtained (r^2=0.83). This shows that when attempting to predict the quantities of Maillard reaction products formed in heated foods, it is necessary to consider all the Maillard precursors and not just those directly related to the products of interest.

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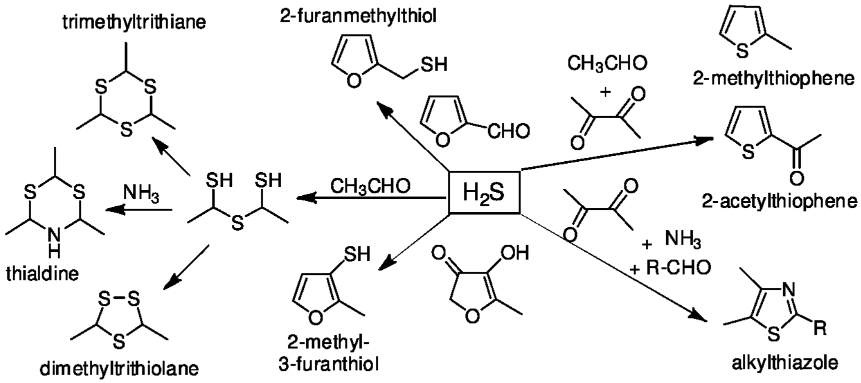
trimethyltrithiane
2-furanmethylthiol
SH
CHO
CH3CHO
+
2-methylthiophene
2-acetylthiophene
NH3
CH3CHO
H2S
thialdine
+ NH3
+ R-CHO
dimethyltrithiolane
2-methyl-
3-furanthiol
alkylthiazole
R

Figure 3. Pathways for the formation of heterocyclic aroma compounds in the reaction of hydrogen sulfide with Maillard derived intermediates.

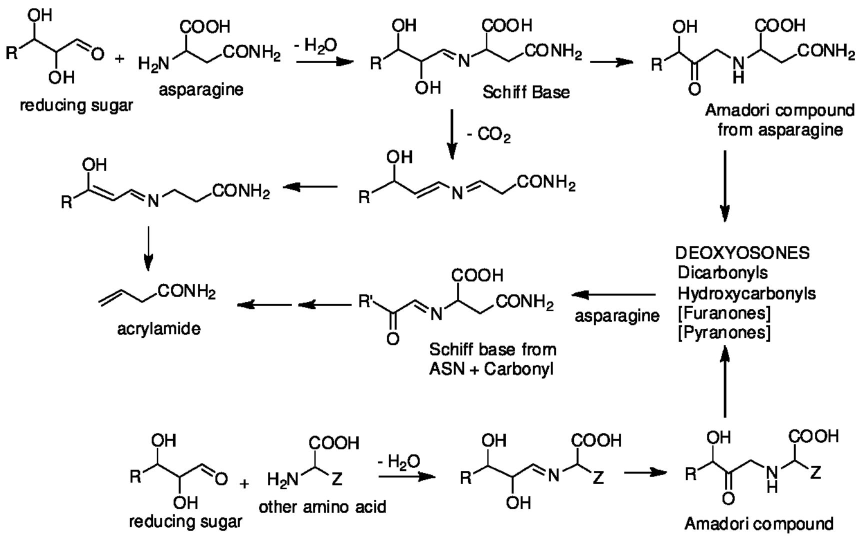
reducing sugar
asparagine
- H2O
Schiff Base
Amadori compound
from asparagine
- CO2
DEOXYOSONES
Dicarbonyls
Hydroxycarbonyls
[Furanones]
[Pyranones]
acrylamide
asparagine
Schiff base from
ASN + Carbonyl
reducing sugar
other amino acid
- H2O
Amadori compound

Figure 4. Routes to the formation of acrylamide from asparagine and reducing sugars in the presence of other amino acids.

Kinetic modeling has been used to predict rates of formation of components of the Maillard reaction, as a function of temperature, pH, water activity/content, and chemical reactivity (*24*). A few papers have reported applications to predict acrylamide formation in heated foods (*21*, *25*), although there have been many more papers just examining the kinetics of asparagine–sugar model systems. However, the latter do not often provide predictions for acrylamide in real foods. The application of kinetic modeling to the Maillard reaction for color and flavor are discussed in several other papers in this book (*26–28*).

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Interaction between the Maillard Reaction and Lipids

The Maillard reaction is just one group of reactions occurring during the cooking of foods that contributes to flavor. Other food components, such as thiamin, tocopherol and carotenoids, can degrade and provide taste and flavor compounds. However, autoxidation of lipids provides the largest source of compounds with flavor potential.

The major class of lipid autoxidation products are saturated and unsaturated aldehydes. The presence of reactive carbonyls in these compounds provides additional intermediates for the Maillard reaction thus giving the potential of modifying the overall profile of Maillard compounds and/or forming other aroma compounds. In heated model systems containing ribose and cysteine, it was demonstrated that the addition of phospholipid gave compounds formed by the interaction of lipid autoxidation products with intermediates of the Maillard reaction (*29*, *30*). Compounds that arise from the interaction of lipid with the Maillard reaction have also been found in the volatiles of cooked foods (*18*). These compounds include O-, N- or S-heterocycles containing long *n*-alkyl substituents. The *n*-alkyl groups are derived from aliphatic aldehydes obtained from lipid oxidation, while amino acids are the source of the nitrogen and sulfur.

Meat volatiles have been found to contain the largest number of such compounds (*31*). Heterocyclic compounds with long *n*-alkyl substituents found in cooked meat include 2-pentylpyridine, and 2-alkylthiophenes, 2-alkyl-(2*H*)-thiapyrans, 2-alkylthiazoles and 2-alkylthiazolines with *n*-alkyl substituents containing between 2 and 15 carbons (Figure 5).

In studies on the polyunsaturated fatty acid (PUFA) composition of lamb and beef, it was observed that meat from animals fed sources of PUFA gave meat with higher PUFA levels (*32*, *33*). This meat was more prone to lipid autoxidation during cooking, resulting in higher concentrations of lipid-derived aldehydes. The cooked meat with higher PUFA also contained higher levels of alkylthiophenes, alkylthiapyrans, alkylthiazoles and alkylthiazolines, confirming that the lipid-derived aldehydes interacted with Maillard intermediates. Some alkylimidazoles and alkyloxazolines were also identified tentatively but their identities were not confirmed (J.S. Elmore, unpublished). The pathway to the alkylthiophenes and alkylthiapyrans was believed to be the reaction of hydrogen sulfide with dienals. To explain formation of alkylthiazoles and alkylthiazolines, it was suggested that dicarbonyls, ammonia and hydrogen sulfide, from the Maillard reaction, reacted with alkanals derived from lipid autoxidation.

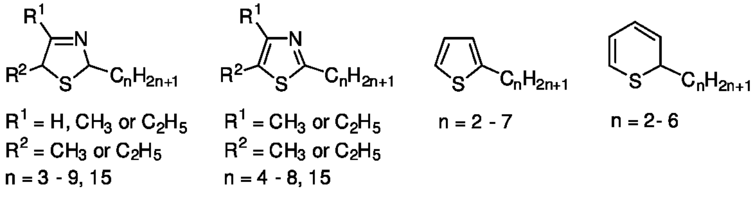

Figure 5. Thiazolines, thiazoles, thiophenes and thiapyrans found in cooked meats resulting from the interaction of lipid-derived aldehydes with the Maillard reaction.

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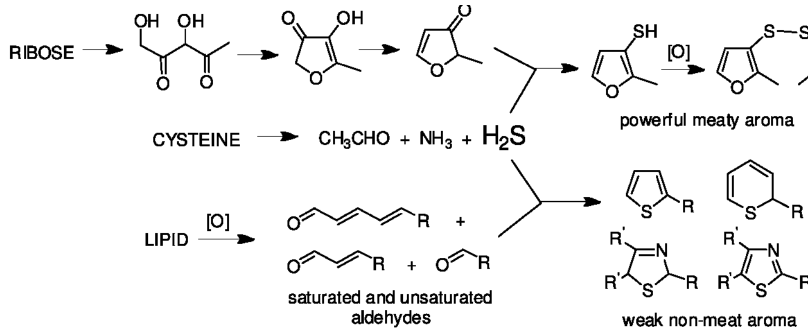

Figure 6. Competition between lipid derived aldehydes and Maillard-derived furanones for available hydrogen sulfide in thermal generation of meat flavor.

The odors of thiazolines and thiazoles with 2-*n*-alkyl substitution were described as slightly fatty, but they did not have low odor threshold values. Thiazolines and thiazoles with 2-methyl or 2-acetyl substituents possess thresholds in the low μg/kg range, but it appears that the larger molecules with long alkyl substituents are not such potent odorants. Similarly, the aromas of the alkylthiapyrans and alkylythiophenes were weak, suggesting that it is unlikely that any of these compounds contribute directly to cooked meat aroma. However, their presence in the meat confirms that lipid–Maillard interactions do take place during the cooking of meat. Such interactions will modify the profile of aroma compounds produced by the Maillard reaction and thus indirectly affect the aroma. Hydrogen sulfide is a key component in the formation of meat-like aroma compounds, such as furanthiols and disulfides. The aldehydes that are produced in meat during cooking will compete with the furanone precursors of these S-containing furans for available hydrogen sulfide (Figure 6). This results in only low concentrations of the potent S-containing furans in meat.

While this might suggest that lipids prevent the production of desirable aroma, in practice only very low concentrations are required to give meaty aroma and it is hypothesized that, through competition for hydrogen sulfide, lipid maintains these compounds at an optimum level in cooked meat. In systems where high levels are found, e.g. reaction mixtures of cysteine and ribose, the aromas can be overpoweringly sulfurous. This is an example of how, in real foods, flavor generation in the Maillard reaction is controlled by other meat components.

Interaction between Maillard Reaction Products and Protein

The importance of compounds such as bis(2-methyl-3-furyl) disulfide and bis(2-furfuryl) disulfide and their corresponding thiols in heated flavors, especially meat and coffee, has been discussed above. It has been found that these compounds will interact with protein, providing another mechanism by which their concentration in meat and other proteinaceous food is controlled. When these two disulfides were added to a meat system (minced beef) and to ovalbumin, a significant proportion of the disulfides were broken down to corresponding thiols (2-methyl-3-furanthiol and 2-furanmethanethiol, respectively) and some were lost completely (*34*, *35*). In addition, small amounts of mixed disulfides, and 2-methyl-3-furyl methyl disulfide and 2-furfuryl methyl disulfide were formed in the meat system. An aqueous blank, which was used as a control, showed no breakdown of the disulfides. Furthermore, it was observed that very little change was seen when the disulfides were added to casein, which does not contain free sulfhydryl groups. In proteins, redox reactions involving interchange of sulfhydryl and disulfide groups within the protein or with external thiol groups are well known (*36*) and such interaction between the furan disulfides and protein sulfhydryl groups provides a clear explanation for their loss in meat systems. As well as having implications for the control of these Maillard reaction products in foods during cooking, this observation is also important in their application in food flavorings.

Other disulfides, including dialkyl disulfides were shown to interact with proteins in a similar way, resulting in large decreases in concentration when they were heated with protein (*37*). This raises other questions about interactions in food between volatiles and food components. Alliaceous vegetables, such as onions and garlic, contain large quantities of di- and tri-sulfides, and these would be expected to interact with protein if heated with a proteinaceous food. Meat is often cooked with onions and changes to the aroma profile are very noticeable. Analysis of the volatiles of onion heated with and without meat showed very large differences in the profiles (Figure 7), with extensive loss of di- and tri-sulfides when meat was present, due to binding to the meat protein (*38*). Although, in this work no evidence of reaction between the Maillard-derived disulfides and onion di- and tri-sulfides was found, this is an interesting area of research which may, in the future, identify novel compounds from such interactions.

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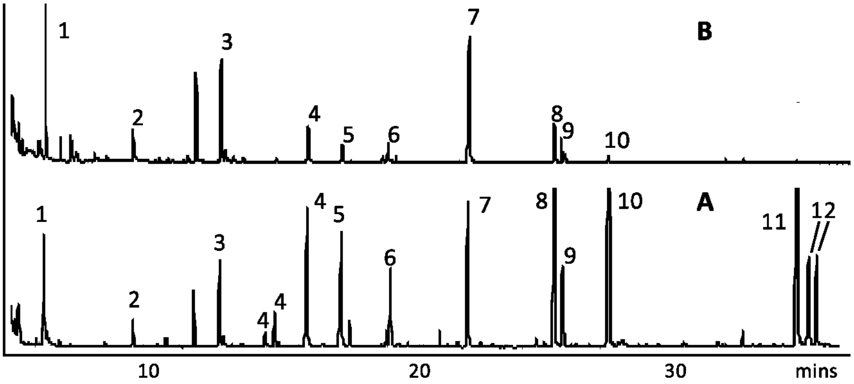

Figure 7. GC-MS analysis of volatiles from onion: A heated without meat; B heated with meat. 1. 1-propanethiol, 2. dimethyl disulfide, 3. 2-methyl-2-pentenal, 4. dimethylthiophenes, 5. methyl propyl disulfide, 6. dimethyl trisulfide, 7. dichlorobenzene (IS), 8. dipropyl disulfide, 9. 1-propenyl propyl disulfide, 10. methyl propyl trisulfide, 11. dipropyl trisulfide, 12. (E/Z)-1-propenyl propyl trisulfides

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Conclusions

For over 60 years the Maillard reaction has been of considerable academic and technological interest to food scientists. Through studies of reactions between individual amino acids and sugars a significant knowledge has been acquired of the products of the reaction, their pathways of formation and their contribution to quality aspects of heated foods, especially flavor. Foods are more complex than model systems since they contain mixtures of many free amino acids and sugars, as well as structural food components and other components that react during cooking. The profile of aroma compounds in a heated food depends on the interaction between Maillard intermediates derived from sugars and amino acids, and these intermediates depend on the relative composition of free amino acids and sugars in the food. In addition compounds derived from lipid oxidation, and other food components, participate in these interactions and help to provide natural control for the Maillard reaction so that recognizable characteristic flavor for a particular food is delivered consistently over wide ranges of cooking conditions.

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